(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 293 274**
A1

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 88400790.7

(22) Date of filing: 31.03.88

(51) Int. Cl.4: C 12 N 15/00
A 61 K 39/29

(30) Priority: 31.03.87 JP 78313/87
04.06.87 JP 140586/87
10.11.87 JP 283990/87

(43) Date of publication of application:
30.11.88 Bulletin 88/48

(84) Designated Contracting States:
CH DE FR GB IT LI NL SE

(71) Applicant: MITSUBISHI CHEMICAL INDUSTRIES LIMITED
5-2, Marunouchi 2-chome Chiyoda-ku
Tokyo 100 (JP)

(72) Inventor: Takahashi, Kazuhiro 303 Corpo Omodaka
29 Ichijoji-Somedono-cho Sakyo-ku
Kyoto City Kyoto Prefecture (JP)

Shibui, Tatsurou
M606-11 San White 6-15-4 Minami-Naruse
Machida City Tokyo (JP)

Kamizono, Michiru
F202-24 San White 2-8-11 Tsuruma
Machida City Tokyo (JP)

Matsui, Rie
A211-23 San White 1-26-31 Ogawa
Machida City Tokyo (JP)

Teranishi, Yutaka
1-14-2 Takane
Sagamihara City Kanagawa Prefecture (JP)

Nakanishi, Shigetada
517-116 Iwakura-Nagatani-cho Sakyo-ku
Kyoto City Kyoto Prefecture (JP)

Kitamura, Naomi
2-272 Yakumo-Higashi-machi
Moriguchi City Osaka Prefecture (JP)

(74) Representative: Gutmann, Ernest et al
S.C. Ernest Gutmann - Yves Plasseraud 67, boulevard Haussmann
F-75008 Paris (FR)

The title of the invention has been amended (Guidelines for Examination in the EPO, A-III, 7.3).
The application is published incomplete as filed (Article 93 (2) EPC). The point in the description or the claim(s) at which the omission obviously occurs has been left blank.

(54) DNA molecules encoding non-A, non-B hepatitis antigens, and their use in producing said antigens.

(57) Provided herein are a DNA fragment which contains a base sequence coding for a non-A non-B hepatitis-specific antigenic protein occurring in cells of the liver affected with non-A non-B hepatitis, an expression vector in which said DNA fragment is inserted into a cloning site present downstream from a promoter thereof, a transformant obtained by introducing said expression vector into a host, and a process for producing said antigenic protein which comprises providing said expression vector, transforming a host with said expression vector, culturing the transformed host and collecing the protein produced therein.

EP 0 293 274 A1

Bundesdruckerei Berlin

Description

DNA FRAGMENTS CODING FOR ANTIGENS SPECIFIC TO NON-A NON-B HEPATITIS, EXPRESSION VECTORS CONTAINING SAID DNA FRAGMENTS, TRANSFORMANTS AND PROCESS FOR PRODUCING SAID ANTIGENS

BACKGROUND OF THE INVENTION

Field of the Invention:

The present invention generally relates to the production of an antigen specific to non-A non-B hepatitis by recombinant DNA technology. More particularly, it relates to a DNA fragment coding for an antigen specifically occurring in a host affected with non-A non-B hepatitis, an expression vector containing such a DNA fragment, a host transformed with such an expression vector, as well as a process for producing said antigen specific to non-A non-B hepatitis by culturing such a transformant.

Description of the Prior Art:

Among viral hepatitises, the viral entities of hepatitis type A and type B have been found and, accordingly, it has now become possible to diagnose such diseases by immunological methods.

Still another type of hepatitis different from the types A and B, which is called non-A non-β type hepatitis, is said to be over 90% of post-transfusion hepatitis: refer to NIPPON RINSHO (Japan Clinic), 35, 2724 (1977); J. Biol. Med., 49, 243 (1976). The pathogenic virus of the non-A non-B type hepatitis, however, has not yet been identified. Only one fact which has already been established is potential infection of human hepatitis type non-A non-B virus to chimpanzee: refer to Lancet I, 459 (1978); ibid., 463 (1978).

Many workers have done various investigations for searching an antigen-antibody system related to the non-A non-B hepatitis by using mainly sera from patients affected with the disease; nevertheless, no definite system has been found. Under these circumstances, the diagnosis of non-A non-B hepatitis should inevitably be effected by so-called exclusion diagnosis: that is, whether or not the hepatitis of a patient is type A or type B or other hepatitis due to a virus known to cause hepatopathy, for example, CMV, HSV, EBV, etc., is first determined; and if not, the patient's hepatitis is diagnosed as non-A non-B type. Thus, such a diagnosis of non-A non-B hepatitis will require much time and labor.

An antigenic protein specific to non-A non-B hepatitis and useful for the direct diagnoses of the hepatitis has been purified from human and chimpanzee hepatocytes affected with non-A non-B hepatitis, and a monoclonal antibody specific to the antigen and useful for the treatment of the non-A non-B hepatitis has also been proposed: refer to Japanese Patent Application Laying-open (KOKAI) Nos. 176856/86 and 56196/86.

A large amount of such an antigenic protein specific to non-A non-B hepatitis should be required when such a protein is to be employed, for example, as a diagnostic agent. However, it is not always appropriate to purify such a large amount of the antigenic protein from chimpanzee hepatocytes affected with non-A non-B hepatitis.

On the other hand, in order to detect a gene coding for a specific antigen of non-A non-B hepatitis by nucleic acid hybridization and, further, to produce such an antigen specific to non-A non-B hepatitis by the recombinant DNA technology, it is essential to obtain a gene fragment coding for the antigenic protein specific to the non-A non-B hepatitis.

SUMMARY OF THE INVENTION

The present inventors have made great efforts to produce such a specific antigenic protein in a large amount by genetic engineering techniques, and finally isolated a gene fragment coding for the antigenic protein specific to non-A non-B hepatitis, said gene fragment being useful for the production of such antigens. Further, the inventors have successfully constructed an expression vector containing said gene fragment. Thus, the present invention has now been attained.

It is an object of the invention to provide a DNA fragment which contains a base sequence coding for an antigen specifically occurring in a host cell affected with non-A non-B hepatitis or an antigenic protein specific to non-A non-B hepatitis having physiological activities equivalent to those of said specifically occurring antigen.

Another object of the invention is to provide an expression vector having said DNA fragment introduced thereinto at a cloning site downstream from a promoter of the vector.

A still another object of the invention is to provide a transformant obtained by transforming a host cell with said expression vector.

A further object of the invention is to provide a process for producing such an antigen specific to non-A non-B hepatitis by culturing said transformant.

BRIEF DESCRIPTION OF THE DRAWINGS

Other objects and advantages of the present invention will be apparent from the following detailed description with reference to the attached drawings, in which:

Figs. 1a-1e show the base sequence coding for an antigenic protein specific to non-A non-B hepatitis;

Fig. 2 shows the base sequence of a hybrid promoter Pac;

Figs. 3a-3c show the base sequence of a cDNA fragment obtained in Example 1 described hereinbelow, together with deduced amino acid sequence;

Figs. 4a-4c show the base sequence of cDNA containing the full length gene sequence of an antigenic protein specific to non-A non-B hepatitis, which cDNA was obtained in Example 2 described hereinbelow, the base sequence 57-1388 thereof coding for the antigenic protein specific to non-A non-B hepatitis;

Fig. 5 schematically illustrates the construction of a plasmid pCV44H;

Fig. 6 schematically illustrates the construction of a plasmid pCV44B; and

Fig. 7 schematically illustrates the construction of a plasmid pCZ44.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be described in detail hereinbelow.

According to one aspect of the invention, a DNA fragment is provided which contains a base sequence coding for an antigenic protein occurring specifically in hepatocytes affected with non-A non-B hepatitis.

Such a DNA fragment of the invention may be prepared in the following manner.

First, a liver tissue specimen derived from a human or chimpanzee individual affected with non-A non-B hepatitis is homogenized in an aqueous solution of guanidinium thiocyanate and then subjected to cesium chloride equilibrium dnsity gradient centrifugation according to Chirgwin et al. method (Biochemistry, 18, 5294-5299 (1979)) to separate total RNA as a precipitate. After separation, the total RNA is purified by phenol extraction and ethanol precipitation.

"Individuals affected with non-A non-B hepatitis" used as sources of liver tissue specimens in the invention may include those affected with so-called type D hepatitis, which has recently been named.

It is known that mRNA of an antigen gene generally has a poly-A chain. Thus, the total RNA is subjected to oligo(dT) cellulose column chromatography in a conventional manner and poly(A)-containing RNA (poly A^+ RNA) is isolated as mRNA material.

A cDNA library corresponding to the poly A^+ RNA is then obtained from the mRNA material according to the random primer method (Y. Ebina et al., Cell, 40, 747-758 (1980)): Thus, a number of DNAs complementary to the mRNA material are randomly synthesized using any primer of e.g. about 6 bases and a reverse transcriptase.

The cDNA is methylated with a DNA methylase, e.g. EcoRI methylase, to protect cleavage sites present in the cDNA capable of being cleaved by a corresponding restriction enzyme, e.g. EcoRI. A DNA linker containing the corresponding restriction enzyme cleavage sites at both ends, e.g. EcoRI linker (CGAATTCG), is added to the methylated cDNA and, then, this cDNA is digested with the restriction enzyme, e.g. EcoRI.

The digested cDNA is then cloned into a cloning vector such as a plasmid or a λ phage. For example, the cDNA may be introduced into EcoRI site of λgt 11 DNA, which is an expression cloning vector: refer to R.A. Young et al., Pro. Natl. Acad. Sci. U.S.A., 80, 1194-1198 (1983). The cDNA will be inserted into the β-gal gene on the λgt 11 phage. Thus, expression of the cDNA can be easily verified by the production of a fused protein with β-galactosidase due to induction of the expression by the lactose operon promoter of said phage when E. coli transfected with said phage is cultured in a medium containing IPTG (Isopropylthio-β-D-galactopyranoside).

The λgt 11 phage incorporating the cDNA is then introduced into E. coli by Tomizawa et al. method in "Experimental Procedures for Bacteriophages", pp. 99-174, published May 30, 1970 by Iwanami Shoten (Japan). The thus transfected microorganism is cultured in an IPTG-containing medium.

The thus formed plaques can be easily selected by an immunological screening method using a monoclonal antibody specifically directed to non-A non-B hepatitis to obtain a desired cDNA. Such a monoclonal antibody which can be used in the immunological screening method may be prepared according to the methods described in Japanese Patent Application Laying-open Nos. 176856/86 and 56196/86. The screening methods used may include the western blotting technique described in these applications.

The plaques positive in the immunological screening test are selected to proliferate the phage by Tomizawa et al. method. DNA is purified from the grown phage by T. Maniatis et al. method in "Molecular Cloning", Cold Spring Harbor Laboratory, pp. 85 et seq. (1982), and cleaved with a suitable restriction enzyme such as EcoRI. The thus purified and digested DNA fragments can be used to determine the base sequence of a desired cDNA segment according to Maxam and Gilbert method in Methods in Enzymology, 65, 499-560 (1980); or alternatively, after further cloning the DNA fragments into M13 phage, the base sequence of such a desired cDNA segment can be determined according to the dideoxy method: Sanger et al., Proc. Natl. Acad. Sci. U.S.A., 74, 5463 (1977).

Thus, a cDNA fragment coding for an antigen specific to non-A non-B hepatitis can be obtained. However, such a DNA fragment may usually be only a portion of the gene coding for the non-A non-B hepatitis-specific antigen.

A full length cDNA coding for such a non-A non-B hepatitis-specific antigen may be obtained in the following manner.

Poly A^+-mRNA is isolated and purified in a manner similar to that described above. From the poly A^+-mRNA a cDNA library is obtained according to Okayama-Berg vector-primer method: Molecular and Cellular Biology, 2, 161-170 (1982).

A plasmid containing such a cDNA thus prepared is used to transform E. coli by any conventional method, for instance, the method D. Hanahan: J. Mol. Biol., 166, 557 (1983). The transformant ampicillin-resistant

strains are collected and screened by the colony hybridization method using the aforementioned DNA fragment as a probe. Such a probe may preferably be prepared by either the strepto-avidin method, or the nick translation method using photobiotinnucleic acids and ^{32}P-nucleic acids.

The thus selected colonies containing a cDNA clone are cultured. Plasmid DNA is obtained from the cultured colony according to Birmboim et al. method (Nucleic Acid Res., 7, 1513 (1979)) and digested with a suitable restriction enzyme. The base sequence of a desired full-length cDNA segment is then determined according to the aforementioned Maxam and Gilbert method or, alternatively, after further cloning the digested DNA into M13 phage or pVC12 plasmid, such a base sequence is determined according to the above described Sanger et al. dideoxy method.

The base sequence of the full length DNA coding for an antigen specific to non-A non-B hepatitis is shown in Fig. 1, in which the symbol "-" just under the base sequence represents a corresponding base complementary to the respective base described just above each of the symbols.

Of course, DNA fragments which can be employed in the invention do not necessarily contain the same base sequence as shown in Fig. 1, but those DNA fragments in which a part of said base sequence shown in Fig. 1 has been substituted by at least one different base or deleted therefrom and those DNA fragments in which one or more additional bases have been added to the base sequence of Fig. 1 may also be included herein provided that such different DNA fragments may code for substances having physiological activities equivalent to those of the non-A non-B hepatitis-specific antigens encoded by the base sequence of Fig. 1.

According to another aspect of the invention, an expression vector is provided in which the aforementioned DNA fragment of the invention is inserted into a cloning site downstream from a promoter of this vector.

The expression vector of the invention contains a promoter in a position capable of controlling the transcription of a DNA fragment coding for a non-A non-B hepatitis-specific antigen obtained by the aforementioned method. The promoters used in the invention may be any promoter capable of expressing the DNA fragment in a host, and preferably of controlling the transcription of the fragment.

When a host used is a microorganism such as Escherichia coli, Bacillus subtilis, etc., the expression vector of the invention may preferably comprise a promoter, a ribosome binding sequence, a gene for a non-A non-B hepatitis-specific antigen, a transcription termination factor, and a gene controlling the promoter.

The promoter used may include those derived from E. coli, phage, etc., for example, tryptophan synthase operon (trp), lactose operon (lac), lipoprotein (lpp), recA, lambda phage P_L, P_R, T5 early gene P_{25}, P_{26} promoter, which may also be prepared by chemical synthesis. Also included herein are hybrid promoters such as tac (trp:lac), trc (trp:lac) and Pac (phage:E. coli) shown in Fig. 2.

The ribosome binding sequence may be derived from E. coli, phage, etc., but preferably may be those synthetically prepared, for example, those containing a consensus sequence such as

AGGAGGTTTAA.
SD sequence

The gene for a non-A non-B hepatitis-specific antigen may be directly employed without any modification. Preferably, an unnecessary base sequence (non-coding region) may be deleted by site-directed mutagenesis: BIO TECHNOLOGY, July, 636-639 (1984).

A transcription termination factor may not always be required in the expression vector of the invention. Preferably, the instant vector may contain a ρ-independent termi nator, for example, lpp terminator, trp operon terminator, ribosomal RNA gene terminator, etc.

The expression vector may be derived from any conventional plasmid. Preferably, it may be derived from such a plasmid as replicating itself in E. coli or Bacillus subtilis, for example, pBR322- or pUB110-derived plasmid.

Desirably, these factors required for expression are arranged in the expression plasmid in the order of the promoter, the SD sequence, the structural gene of a non-A non-B hepatitis-specific antigen, and the transcription termination factor from 5′ to 3′. A repressor gene required to control the transcription, a marker gene such as drug-resistant gene, and a plasmid replication origin may be arranged in any order in the expression vector.

The expression vector of the invention may be introduced into a host by any conventional method for transformation of E. coli, e.g., one described in Molecular Cloning, 250-253 (1982), or of Bacillus subtilis, e.g., one described in Molec. Gen. Genet., 168, 111-115 (1979) or Proc. Nat. Acad. Sci. U.S.A., 44, 1072-1078 (1958).

The resulting transformant may be cultured in any conventional medium, e.g. one described in Molecular Cloning, 68-73, (1972), at a temperature in the range of 28 to 42°C in both cases of E. coli and Bacillus subtilis. Preferably, it may be cultured at a temperature in the range of 28 to 30°C where no expression of heat shock proteins may be induced.

The desired protein thus produced may be easily purified from the host in conventional procedures. For example, the host cell may be crushed by lysozyme-surfactant or ultra-sonication, and the insoluble fractions which contain the desired non-A non-B hepatitis-specific antigen may be then collected by centrifugation,

solubilized in a surfactant such as 0.01% SDS, and subjected to column chromatography using a monoclonal antibody (Japanese Patent Application Laying-open (KOKAI) Nos. 56196/86 and 176856/86.

When an eukaryotic cell such as an animal cell is employed as a host, the expression vector of the invention is preferably as follows:

The promoters used in the vector of the invention for the expression in eukaryotic cells may herein include SV40 early and late promoters; promoters of apolipoprotein E and A-I genes; promoter of heat shock protein gene (Proc. Natl. Acad. Sci. U.S.A., 78, 7038-7042 (1981)); promoter of metallothionein gene (Proc. Natl. Acad. Sci. U.S.A., 77, 6511-6515 (1980)); HSV TK promoter; adenovirus promoter, such as Ad2 major late promoter (Ad2 MLP); LTR (long terminal repeat) of retrovirus; etc. SV40 promoter and promoter of metallothionein gene are preferred.

The expression vector of the invention may contain a splice sequence comprising 5′ splice junction donor site, an intron and 3′ splice junction acceptor site. A common base sequence is found at all the splice junction sites (exonintron junction sites); so-called GT/AG rule that any intron region always starts from two bases GT at the donor site and terminates at two bases AG of the acceptor site has been established.

The expression vector of the invention may contain one or more splice sequences as mentioned just above. Such splice sequences may be positioned upstream or downstream of the structural gene for a non-A non-B hepatitis-specific antigen.

Illustrative examples of such splice sequences may include those DNA sequences found in exons 2 and 3 of rabbit β-globin gene (Science, 26, 339 (1979)) and mouse methallothionein-I gene containing the promoter, exons 1, 2 and 3 and introns A and B of methallothionein gene (Proc. Natl. Acad. Sci. U.S.A., 77, 6513 (1980)). The 5′ and 3′ splice sites may be derived from the same or different gene; for example, a sequence in which 5′ splice site contained in adenovirus DNA is linked to 3′ splice site derived from the gene of Ig variable region can be employed.

The expression vector of the invention also contains a polyadenylation site downstream from the structural gene of a non-A non-B hepatitis-specific antigen. Illustrative examples of the polyadenylation sites may include those derived from SV40 DNA, β-globin gene or methallothionein gene. A combined site of the polyadenylation sites of β-globin gene and SV40 DNA may be employed in the invention.

The expression vector of the invention may also contain a dominant selective marker permitting the selection of transformants. Selective markers which can be used herein may include DHFR gene imparting MTX (methotrexate) resistance to a host; tk gene of herpes simplex virus (HSV) which permits selection of tk^- strains transformed therewith in HAT medium; the gene for aminoglycoside 3′-phosphotransferase from E. coli transposon Tn5, which imparts to a host the resistance against 3′-deoxystreptamine antibiotic G418; bovine papilloma virus gene permitting morphological discrimination by piled up growth; and aprt gene.

Alternatively, animal cells transformed with the expression vector of the invention may be selected by the cotransformation even though no selective marker is present in the vector. For this purpose, an animal cell is cotransformed with both the expression vector and a plasmid or other DNA containing a gene for such a selective marker and selected by a phenotypic trait of the gene.

Advantageously, the expression vectors may also contain a plasmid fragment having an origin of replication derived from a bacterium such as E. coli, since such vectors can be cloned in bacteria. Such plasmids may include pBR322, pBR327, pML, etc.

Illustrative examples of plasmid vectors used as sources of the expression vectors according to the invention may include pKCR (Proc. Natl. Acad. Sci. U.S.A., 78, 1528 (1981)), which contains SV40 early promoter, the splice sequence and polyadenylation site derived from rabbit β-globin gene, the polyadenylation site from SV40 early region, and the origin of replication and ampicillin resistant gene from pBR322; pKCR H2 (Nature, 307, 605 (1984)), in which the pBR322 portion of pKCR has been substituted by pBR327 fragment and the EcoRI site present in the exon 3 of rabbit β-globin gene has been converted into HindIII site; and pBPV MT1 containing BPV gene and methallothionein gene (Proc. Natl. Acad. Sci. U.S.A., 80, 398 (1983)).

Animal cells transformed with the expression vector of the invention may include CHO cells, COS cells, and mouse L cells, C127 cells and FM3A cells.

The introduction of the expression vector of the invention into an animal cell may be carried out by transfection, microinjection, etc. Most often, the transfection may employ $CaPO_4$: Virology, 52, 456-467 (1973).

Animal cells transformed by introducing the expression vector of the invention may be cultured in a suspension or solid medium by conventional methods. The culture medium used is most often MEM, RPMI1640, etc.

Proteins produced in the transformed animal cells can be separated and purified in the almost same manner as in the case of microorganisms aforementioned.

As stated, the invention provides a transformant cell obtained by introducing the expression vector of the invention into a host cell.

Also provided according to the invention is a process for producing a non-A non-B hepatitis-specific antigen comprising culturing said transformant and collecting the produced and accumulated antigen.

As stated previously, a large amount of an antigenic protein specific to non-A non-B hepatitis is required when such a protein is to be utilized as a direct diagnostic agent. According to the present invention, such an antigenic protein can be produced with a low cost and a large scale without use of infected chimpanzee hepatocytes. Prior to the present invention, it has been difficult obtain such a large amount of a non-A non-B hepatitis-specific antigenic protein from hepatocytes of chimpanzees affected with non-A non-B hepatitis.

Further, the DNA fragment coding for an antigenic protein of non-A non-B hepatitis virus according to the present invention will be useful as a probe for detecting the gene of said antigenic protein by nucleic acid hybridization.

EXAMPLES

The following examples will be given by way of illustration but these examples in no way limit the scope of the invention without departing the concept thereof.

EXAMPLE 1: Preparation of cDNA Fragment Coding for Antigenic Protein Specific to Non-A Non-B Hepatitis

Poly(A)-containing RNA was prepared from chimpanzee liver according to the guanidine thiocyanate-lithium chloride method: Cathala et al., DNA, 2, 329 (1983).

The infected liver (5 g) was taken out from a chimpanzee affected with non-A non-B hepatitis and immediately frozen by liquid nitrogen. The frozen liver was added into a Waring blender together with liquid nitrogen and ground at 3,000 rpm for 2 minutes. The ground liver specimen was further ground by a Teflon homogenizer at 5 rpm in 100 ml of a solution: 5 M guanidine thiocyanate, 10 mM EDTA, 50 mM Tris-HCl (pH 7), 8% (v/v) β-mercaptoethanol. The thus solubilized material (20 ml) was slowly placed on 5.7 M CsCl solution (10 ml) contained in a centrifuge tube and centrifuged at 27,000 rpm for 20 hours in Hitachi RPS 28-2 rotor. The thus precipitated RNA was collected and dissolved in 10 ml of a solution: 0.1% sodium laurylsulfate, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5). The RNA was extracted with phenol-chloroform and re covered by ethanol precipitation.

The thus obtained RNA (about 3.95 mg) was dissolved in 1 ml of a solution: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The solution was incubated at 65°C for 5 minutes, and 5 M NaCl (0.1 ml) was added. The resulting mixture was subjected to chromatography on an oligo(dT) cellulose column (column volume of 0.5 ml, P-L Biochemical). The thus adsorbed poly(A)-containing mRNA was eluted with a solution: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. There was obtained about 100 μg of poly(A)-containing mRNA.

The thus obtained poly(A)$^+$ mRNA (10 μg) was dissolved in 50 μl of RT buffer: 20 mM Tris-HCl (pH 8.8), 0.1 M KCl, 12 mM $MgCl_2$, 2 mM $MnCl_2$. To this solution, there was added 8 μg of random primer $d(N)_6$ (P-L Biochemical). The resulting mixture was heated at 95°C for 3 minutes to denature the materials, which was then cooled gradually to room temperature to anneal the random primer with the mRNA. To the annealed mixture, there were aded 10 mM 4NTP (10 μl) and reverse transcriptase (225 units) from TAKARA SHUZO (Japan), and then water was added so as to make the total volume of the mixture to 100 μl. Reaction was allowed to proceed at 42°C for one hour.

To the reaction mixture (50 μl), there were added 10 mM NAD (2 μl), 10 mM 4dNTP (10 μl), RNase H (5 units), E. coli ligase (1 unit), E. coli DNA polymerase I (6.3 units), and 10 × T4 DNA ligase buffer (10 μl; 0.1 M Tris-HCl, pH 7.5, 0.1 M DTT, 60 mM $MgCl_2$) to make the total volume to 100 μl. The mixture was allowed to react at 37°C for one hour to synthesize a double stranded DNA.

The thus obtained double stranded DNA was extracted with an equal volume of water-saturated phenol. Phenol in the aqueous layer was removed with the aid of ether followed by ethanol precipitation. The precipitate thus obtained was dissolved in 50 μl of water, and 10 × T4 DNA polymerase buffer (10 μl; 0.33 M Tris-acetic acid, pH 7.9, 0.66 M potassium acetate, 0.1 M magnesium acetate, 5 mM DTT), 10 mM 4dNTP (10 μl), and T4 DNA polymerase (6 units) were added to make the total volume to 100 μl. The mixture was reacted at 37°C for one hour. There was obtained a double stranded DNA having blunt ends, which was then extracted with phenol to remove proteins and purified by ethanol precipitation as described above. The thus purified DNA was then air dried.

To the purified DNA, there were added 50 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, 5 mM DTT (20 μl), 100 μM S-adenosyl-L-methionine (2 μl), and 1.8 mg/ml EcoRI methylase (0.2 μl). Reaction was effected at 37°C for 15 minutes, whereby methylating the EcoRI restriction enzyme cleavage site on the DNA fragment. The reaction mixture was then heated at 70°C for 15 minutes to deactivate the enzyme.

To the reaction mixture, there was added 3′-phosphorylated EcoRI linker (GGAATTCC) in an amount of 100 molecules thereof per molecule of the synthetic DNA. There were further added 10 × T4 DNA ligase buffer (5 μl; 0.5 M Tris-HCl, pH 7.5, 60 mM $MgCl_2$, 10 mM DTT), 0.1 M ATP (5 μl), and T4 DNA ligase (5 units) to make the total volume to 50 μl. The resulting reaction mixture was reacted at 4°C for 16 hours followed by heating at 70°C for 10 minutes to deactivate the enzyme. Then, 10 × EcoRI buffer (10 μl; 15 M Tris-HCl, pH 7.5, 0.5 M NaCl, 60 mM $MgCl_2$), and EcoRI (100 units) were added to make the total volume to 100 μl, and the reaction mixture was reacted at 37°C for 2 hours to cut the linker. The reaction mixture was passed through Bio Gel A-50 (0.2 cm × 32 cm, Bio RAD). Elution was effected by a buffer: 10 mM Tris-HCl (pH, 7.5), 6 mM $MgCl_2$. Excess EcoRI linker was removed and, thus, a double stranded cDNA having EcoRI sites at both ends thereof was purified.

To the thus obtained double stranded cDNA fragment having EcoRI sites at both ends, there were added gt 11 DNA (10 μg) cleaved with EcoRI, 10 × T4 DNA ligase buffer (10 μl) as described above, 0.1 M ATP (10 μl), and T4 DNA ligase (10 units) to make the total volume to 100 μl. The mixture was reacted at 4°C for 16 hours. Thus, said double stranded cDNA fragment was inserted into λgt 11 DNA.

The λ phage packaging kit (PROMEGA, Biotech) was used to introduce said DNA into λ phage particle. The procedures for packaging were effected according to the instructions of the kit.

The λgt 11 phage having said DNA packaged thereinto was used to transfect E. coli strain Y1090 to form

plaques according to the conventional Tomizawa et al. methods described in "Experimental Procedures for Bacteriophages", pp. 99-174, published May 30, 1970 by Iwanami Shoten (Japan). Among about 200,000 plaques, one positive clone was selected by immunological screening as described hereinbelow. A monoclonal antibody used in the immunological screening was prepared by the method described in Japanese Patent Application Laying-open (KOKAI) No. 176856/86.

E. coli Y1090 (R.A. Young et al., Pro. Natl. Acad. Sci. U.S.A., 80, 1194-1198 (1983), which had been transfected with λgt 11, was inoculated in a petri dish together with soft agar held at 42°C. The transfected cell was allowed to stand at 42°C for 5 hours. A nitrocellulose filter (S & S, BA-83, pore size of 0.2 μm) containing 10 mM IPTG was placed on the cell in the dish and incubation was effected at 37°C for 3-4 hours. This nitrocellulose filter was lightly rinsed with TBS buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl), immersed in the TBS buffer (400 ml) containing 3% gelatine and shaked at 40°C for one hour. Thus, the nitrocellulose filter was blocked. Then, a monoclonal antibody (OD_{280} = 4.3) directed to a non-A non-B hepatitis-specific antigen was added to TBS buffer containing 1% gelatine with a dilution of 1/400. This mixture was put into a vinyl bag together with the filter in a proportion of 2 ml of the mixture per filter, and reaction was allowed to proceed at room temperature for 16 hours. The reaction mixture was three times washed with TBS buffer (400 ml) containing 0.05% Tween 20 over 10 minutes. A labelled secondary antibody, anti-mouse IgG-PAP (horseradish peroxidase, Bio Rad) was added to TBS buffer containing 1% gelatine with a dilution of 1/1,000. This mixture and the filter were put into a vinyl bag with a proportion of 2 ml of the mixture per filter. Reaction was allowed to proceed at room temperature for 2 hours. The reaction mixture was three times washed with TBS buffer (400 ml) containing 0.05% Tween 20 over 10 minutes, in the same manner as described above. Color development was effected by dipping the filter and 4-chloro-1-naphthol (12 mg, Bio Rad) into 20 ml of TBS buffer containing hydrogen peroxide. After completion of the color development, the filter was thoroughly washed with water and put into a vinyl bag containing water. The bag was stored in a dark and cold place.

Thus, one positive plaque was obtained. The plaque was three times subjected to single plaque isolation. In each time, immunological screening was effected in the same manner as described above, verifying that the plaque was in fact positive.

The phage was then cultured in a large scale to purify the DNA in the following manner: First, E. coli Y1090 was cultured overnight in 10 ml of NZ medium prepared by adding NZ amine (10 g), NaCl (5 g) and 5 mM $MgCl_2$ to one liter of water followed by adjusting the pH to 7.2. The culture (1 ml) was transfected with the phage, with the m.o.i. (multiplicity of infection) being 0.1. The transfected culture was allowed to stand at 37°C for 10 minutes and then transferred to one liter of NZ medium. Shaking culture was effected at 37°C for 7-8 hours until the cells were lysed. Chloroform (5 ml) was added to the culture and shaking was continued for additional 30 minutes. The culture was subjected to centrifugation at 6,500 rpm for 10 minutes to remove cell debris.

NaCl (29 g) and polyethylene glycol (70 g) were added to and thoroughly dissolved in the obtained supernatant, and the solution was allowed to stand at 4°C overnight. The precipitate was collected by centrifugation at 6,500 rpm for 20 minutes, drained thoroughly, and dissolved in 20 ml of TM buffer: 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ DNase I and RNase A were added to the solution, both with a concentration of 10 μg/ml, and the reaction was effected at 37°C for one hour. Chloroform (20 ml) was then added to the reaction mixture and stirred; thus, polyethylene glycol was distributed in the chloroform layer which was then separated from the aqueous layer. This aqueous layer was ultra- centrifuged at 28,000 rpm for 60 minutes. Thus, a pellet of phage particles was obtained.

This pellet was dissolved in TM buffer (1 ml) and subjected to CsCl density gradient centrifugation at 33.000 rpm for 20 hours. The resultant fraction containing the phage particles (ρ = 1.45-1.50) was dialyzed overnight against TM buffer. Proteinase K was added to the dialyzate in an amount of 100 μg/ml and reaction was effected at 37°C for one hour. Thereafter, an equal volume of water-saturated phenol was added and phenol-extraction was gently effected. After centrifugation at 6,500 rpm for 10 minutes, the aqueous layer was removed, put into a dialysis tube, and dialyzed overnight against water at 4°C. Thus, about 5 mg of DNA was obtained.

Cleavage reaction of this DNA (100 μg) with EcoRI (100 units) in the aforementioned buffer (100 μl) at 37°C revealed that two cDNA segments of 390 bp and 345 bp were inserted into the phage DNA.

These two EcoRI fragments were re-cloned into EcoRI site of a cloning vector pUC 119. Base sequences of these DNA fragments were determined by the dideoxy method using commercially available primers CAGGAAACAGCTATGAC and AGTCACGACGTTGTA, respectively. The base sequence of the linking portion between these two DNA fragments was similarly determined by cutting this cDNA fragment at BamHI and EcoRV sites present therein with corresponding specific restriction enzymes, inserting the resulting BamHI-EcoRV DNA fragment between BamHI and SmaI sites of the plasmid pUC 119, and sequencing the fragment by the dideoxy method.

The base sequence of said cDNA fragment is shown in Fig. 3. This was a partial cDNA fragment of a gene coding for an antigenic protein specific to non-A non-B hepatitis.

EXAMPLE 2: Preparation of cDNA Containing the Full Length Gene Sequence

Messenger RNA was prepared as described in Example 1 and cDNA was synthesized using Okayama vector according to the conventional method described in Molecular Cloning, p. 211 et seq. The procedures used to synthesize cDNA were as follows:

To 300 μl of a solution (10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl), there were added 400 μg of

pCDV 1 (Okayama and Berg, Mol. Cell. Biol., 3, 280 (1983)) and 500 units of KpnI (TAKARA SHUZO, Japan), all restriction enzymes used hereinafter having been manufactured by TAKARA SHUZO (Japan) unless otherwise noted. Reaction was effected at 37°C for 6 hours to cut the plasmid at KpnI site therein. After phenol-chloroform extraction, ethanol precipitation was effected to recover DNA.

The DNA (about 200 µg) cleaved with KpnI was added to 200 µl of a solution which was obtained by adding dTTP in a concentration of 0.25 mM to a buffer (TdT buffer): 40 mM sodium cacodylate, 30 mM Tris-HCl (pH 6.8), 1 mM $CaCl_2$, 0.1 mM dithiothreitol (DTT). Further, 81 units of terminal deoxynucleotidyl transferase (TdT, manufactured by P-L Biochemicals) was also added. Reaction was effected at 37°C for 11 minutes. Thus, a poly(dT) chain (about 67 deoxythymidylic acid residues) was added to the 3′ end at the KpnI-cleaved site of pCDV 1. After phenol-chloroform extraction and ethanol-precipitation, about 100 µg of pCDV 1 DNA to which poly(dT) chain had been added was recovered from the reaction mixture.

The thus obtained DNA was added to 150 µl of a buffer (10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl), and HpaI (360 units) was also added, followed by reaction at 37°C for 2 hours. The reaction mixture was subjected to electrophoresis on agarose gel to separate and recover about 3.1 Kbp DNA fragment. Thus, there was obtained about 60 µg of poly(dT)-containing pCDV 1.

The thus obtained DNA was dissolved in 500 µl of a solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), incubated at 65°C for 5 minutes, and cooled on ice. After adding 5 M NaCl (50 µl), the mixture was subjected to chromatography on oligo(dA) cellulose column (Colaborative Research). DNA having a poly(dT) chain of sufficient length was adsorbed on the column and eluted with a solution: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. Thus, there was obtained 27 µg of pCDV 1 to which poly(dT) chain had been added, abbreviated hereinafter as vector primer.

A linker DNA was prepared in the following manner: To 200 µl of a solution (10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 50 mM NaCl), there were added about 14 µg of pL 1 (Okayama and Berg, Mol. Cell. Biol., 3, 280 (1983)) and 50 units of PstI. Reaction was effected at 37°C for 4 hours to cut the pL 1 DNA at PstI site. Phenol-chloroform extraction and ethanol precipitation of the reaction product gave about 13 µg of pL 1 DNA cleaved at PstI site.

The thus obtained DNA (about 13 µg) was added to 50 µl of the TdT buffer containing dGTP at a final concentration of 0.25 mM, and 54 units of TdT (P-L Biochemicals) was also added. The mixture was incubated at 37°C for 13 minutes to add a (dG) chain (about 14 deoxyguanylic acid residues) to the 3′ end at the PstI-cleaved site of pL 1. After phenol-chloroform extraction, DNA was recovered by ethanol precipitation.

The thus obtained DNA was added to 100 µl of a buffer (10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 60 mM NaCl), and 80 units of HindIII was also added. The mixture was incubated at 37°C for 3 hours to cut the pL 1 DNA at HindIII site. The reaction product was fractionated by agarose gel electrophoresis. About 0.5 Kb DNA fragment was recovered by the DEAE paper method: Dretzen et al., Anal. Biochem., 112, 295 (1981). Thus, there was obtained an oligo(dG) chain-containing linker DNA, hereinafter abbreviated simply as linker DNA.

The aforementioned poly(A)$^+$ RNA (about 2 µg) prepared in the same manner as in Example 1 and the vector primer (about 1.4 µg) were dissolved in 22.3 µl of a solution: 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 0.3 mM DTT, 2 mM dNTP (dATP, dTTP, dGTP and dCTP) and 10 units of ribonuclease inhibitor (P-L Biochemicals). To the solution, there was added 10 units of reverse transcriptase manufactured by SEIKAGAKU KOGYO (Japan). Incubation was effected at 37°C for 40 minutes to synthesize a DNA complementary to the mRNA. After phenol-chloroform extraction and ethanol precipitation, the vector primer DNA to which a double stranded RNA-DNA had been added was recovered.

The thus obtained vector primer DNA containing RNA-DNA double stranded chain was dissolved in 20 µl of TdT buffer containing 60 µM dCTP and 0.2 µg poly(A). After adding 14 units of TdT (P-L Biochemical), the mixture was incubated at 37°C for 8 hours to add a (dC) chain of 12 deoxycytidylic acid residues to the 3′ end of the cDNA. The reaction product was extracted with phenol-chloroform and precipitated with ethanol to recover a cDNA-vector primer DNA to which a (dC) chain had been added.

The thus obtained (dC) chain-containing cDNA-vector primer DNA was dissolved in 400 µl of a solution (10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 60 mM NaCl), and 20 units of HindIII was also added. The mixture was incubated at 37°C for 2 hours to cut the DNA at HindIII site. The reaction product was extracted with phenol-chloroform and precipitated with ethanol. Thus, there was obtained 0.5 pmole of a (dC) chain-containing cDNA-vector primer DNA.

The thus obtained (dC) chain-containig cDNA-vector primer DNA (0.08 pmole) and the aforementioned linker DNA (0.16 pmole) were dissolved in 40 µl of a solution: 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA. The resulting solution was incubated at 65°C for 10 minutes, at 42°C for 25 minutes, and then at 0°C for 30 minutes. The reaction mixture was adjusted to 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1 M KCl and 0.1 mM β-NAD in a total volume of 400 µl.

To the reaction mixture, there was added 10 units of E. coli DNA ligase (New England Biolabs), followed by incubation overnight at 11°C. After adjusting the concentrations of dNTP and β-NAD in the reaction mixture to 40 µM and 0.15 mM, respectively, by supplementing necessary reagents, 5 units of E. coli DNA ligase, 7 units of E. coli DNA polymerase I (P-L Biochemicals) and 2 units of E. coli ribonuclease H (P-L Biochemicals) were added to the reaction mixture. The mixture was incubated at 12°C for one hour and then at 25°C for one hour.

In the course of the above reactions, a recombinant DNA containing the cDNA was cyclized and the RNA portion of the RNA-DNA double stranded chain was substituted by DNA. Thus, a desired recombinant plasmid containing a complete double-stranded DNA was produced.

The recombinant plasmid was used to transform competent cells of E. coli strain MC1064 prepared by conventional methods. Approximately 50,000 transformants were fixed on a nitrocellulose filter. These colonies were screened according to the colony hybridization method described in Molecular Cloning, Cold Spring Harbor Laboratory, p. 329 et seq. (1982) using the cDNA fragment obtained in Example 1 as a ^{32}P-labelled probe. Thus, three clones showed strong hybridization at 42°C.

These positive clones were analyzed in detail by Southern method: J. Mol. Biol., 98, 503 (1975). There was obtained the desired full length cDNA of a gene coding for an antigenic protein specific to non-A non-B hepatitis. The base sequence of the cDNA is shown in Fig. 4.

The expression vector containing the full length cDNA was designated as pCDVCL-I.

EXAMPLE 3: Preparation of Expression Vector and Transformant and Expression of Specific Antigen

A. Preparation of Expression Vector and Transformant

I) Modification of N-terminus (Fig. 5):

i) In 100 µl of a buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$), pCDVCL-I (5 µg) was digested with PvuI (10 units) at 37°C for 2 hours. The reaction mixture was heated at 75°C for 15 minutes to deactivate the enzyme, dialyzed against water, and dried. The cleaved plasmid DNA was treated with T4 DNA polymerase (4 units) in 40 µl of a system: 33 mM Tris-acetic acid (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate and 0.5 mM dithiothreitol, to which 2 mM 4-deoxytriphosphate had been added; thus, the 3′ protruding end of the plasmid DNA was filled in to produce a blunt end. The thus treated mixture was heated at 70°C for 10 minutes to deactivate the enzyme, dialyzed against water, and dried. The thus obtained plasmid DNA was then stored in the form of an aqueous solution (50 µl). This plasmid DNA fragment is hereinafter designated as Fragment I.

ii) On the other hand, pCDVCL-I (20 µg) was digested with NcoI and HindIII (each 20 units) at 37°C for 2 hours in 100 µl of a buffer: 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM $MgCl_2$. The plasmid DNA was subjected to 5% acrylamide gel electrophoresis at 10 V/cm for 1.5 hours in a buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA. The gel was stained with 0.05% aqueous ethidium bromide solution and two gel slices corresponding to DNA fragments of larger molecular weights were excised from the gel under ultraviolet radiation at 340 nm. The gel slices were crushed by means of a glass rod, suspended into 4 ml of a buffer for DNA extraction (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% sodium laurylsulfate), and allowed to stand overnight at 37°C to extract DNA from the gel. The materials were subjected to centrifugation at 10,000 rpm for 15 minutes to eliminate larger gel pieces, and passed through a glass filter to remove smaller gel pieces. The DNA was purified by effecting ethanol percipitation three times and stored in the form of an aqueous solution (200 µl). This plasmid DNA fragment is hereinafter designated as Fragment II.

iii) A primer of the DNA portion to be modified as shown below (51 bases) was synthesized by a DNA synthesizer, NIKKAKI (Japan), Applied Biosystem MODEL 380A. The synthesized DNA was overnight reacted with concentrated aqueous ammonia at 55°C to deprotect and purified by reversed HPLC before use.

```
                                        HindIII
Primer                  ACAACAGATCTAAGCTTATGGCAGTTACAACAAGATTAA
                              x  x x          x      xx x  x
(Original sequence) (----------A--A-G---------G-----TC-C--G-

                        CATGGTTGCATG  wherein x represents a
                                      base substitution.
                        ------------)
```

The synthetic primer (150 pmole) was treated with T4 polynucleotide kinase (20 units) in 10 µl of a kinase buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol) to phosphorylate the 5′ end thereof.

iv) Fragment I (0.05 pmole), Fragment II (0.05 pmole) and 5′-phosphorylated primer (45 pmole) were added to 12 µl of 5 × polymerase-ligase buffer (0.5 M NaCl, 32.5 mM Tris-HCl, pH 7.5, 40 mM $MgCl_2$, 5 mM β-mercaptoethanol) to make the total volume of the mixture 34.8 µl. The mixture was boiled at 100°C for 3 minutes, immediately after which it was placed in a thermostat at 30°C and allowed to stand for 30 minutes. The mixture was allowed to stand at 4°C for 30 minutes and then on ice for 10 minutes to form a heteroduplex.

To an aqueous solution (11.6 µl) containing the heteroduplex, there were added 2.5 mM 4-deoxynucleotide

triphosphate (2 µl), 10 mM ATP (2 µl), Klenow enzyme (2 units) and T4 DNA ligase (0.5 units) to form a mixture of 20 µl in total volume. The mixture was reacted overnight at 16°C to cyclize the DNA.

An aqueous solution (2 µl) containing the circular DNA was used to transform E. coli HB101 strain according to conventional methods. Plasmids were separated from the transformant and purified in conventional manners. The plasmid was cleaved with restriction enzyme HindIII and subjected to 5% acrylamide gel electrophoresis. Thus, two separate fragments were collected as desired modified, variant plasmids. Since resulting variant plasmids might often be admixed with original wild-type plasmids, the thus obtained variant plasmids were again employed to transform E. coli HB101 so as to purify the plasmid.

Thus, a purified plasmid pCV44H was obtained (Fig. 5).

II) Modification of C-terminus (Fig. 6):

i) Plasmid pCDVCL-I (5 µg) was treated in the same manner as in I) I) described above to produce Fragment I.

ii) Plasmid pCDVCL-I (20 µg) was treated in ths same manner as in I) ii) described above except that NcoI and NsiI (each 5 units) were employed. Thus, Fragment II was produced.

iii) In the same manner as in I) iii) described above, the following primer (46 bases) was synthesized and the 5′ end thereof was phosphorylated.

```
                                        BglII   SalI
Primer                  GCACAAGGAAAAAAATGAGATCTGTCGACGGTTCACGTA
                                        xxxxxxxxxxxxx
(Original sequence) (----------------AGATATGTGAA*A----------

                        AATTTCC  wherein x represents a base
                                 substitution and * represents
                        -------) an addition.
```

iv) The Fragment I and II and the 5′-phosphorylated primer obtained above in II) I) to iii) were treated in the same manner as in I) iv) described above. Thus, plasmid pCV44B was obtained (Fig. 6).

III) Introduction of cDNA coding for specific antigen into expression vector (Fig. 7):

I) In 100 µl of a buffer H (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$), 10 µg (about 3 pmole) of pCV44H was cut with HindIII (20 units) and SacI (20 units) at 37°C for 2 hours. The reaction mixture was subjected to 5% acrylamide gel electrophoresis. Thus, a 467 bp DNA fragment coding for the N-terminus of the specific antigen was separated and purified. This fragment is hereinafter designated as Fragment N.

ii) In 100 µl of the buffer H, 10 µg (about 3 pmole) of pCV448 was cleaved with BglII (20 units) and SacI (20 units) at 37°C for 2 hours. The reaction mixture was subjected to 5% acrylamide gel electrophoresis to isolate and purify a 836 bp DNA fragment coding for the C-terminus of the specific antigen. The thus obtained fragment is hereinafter designated as Fragment C.

iii) In 20 µl of buffer H, 2 µg (about 1 pmole) of an expression vector pUSΔH was cut with HindIII (2 units) and BglII (2 units) at 37°C for 2 hours. The reaction mixture was extracted with an equal volume of water-saturated phenol to remove proteins. After extracting the phenol with ether, the reaction mixture was dialyzed against water to desalt, and concentrated by a vacuum pump. Thus, there was obtained 10 µl of an aqueous solution containing an expression vector fragment HB.

iv) Fragment N (0.5 pmole), Fragment C (0.5 pmole) and the expression vector fragment HB (0.1 pmole) were mixed and reacted with T4 DNA ligase (1 unit) at 4°C for 16 hours in 10 µl of a buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 6 mM $MgCl_2$, 1 mM ATP). The reaction mixture (3 µl) was used to transform commercially available E. coli JM109 competent cell according to conventional methods. The resulting transformants were selected in L broth plate (bactopeptone 10 g, yeast exstract 5 g, NaCl 10 g, agar 15 g per liter) containing 20 µg/ml ampicillin. Thus, there was obtained an expression vector pCZ44 containing the specific antigen gene inserted thereinto (Fig. 7).

B. Expression of Specific Antigen

E. coli strain JM109 possessing pCZ44 was cultured overnight at 30°C in L broth. The culture was inoculated in a fresh L broth with a dilution of 1/50 and cultured with shaking at 30°C for 2 hours. After IPTG (Isopropylthio-β-D-galactopyranoside) was added to the medium in a concentration of 2 mM, shaking culture was continued at 30°C for further 3 hours. The cells were collected by centrifugation at 6,500 rpm for 10 minutes and suspended in a buffer (0.9% NaCl, 10 mM Tris-HCl, pH 7.5) to store.

C. Verification of Expression of Specific Antigen

The thus obtained cell culture (0.3 ml) was subjected to 10% SDS polyacrylamide gel electrophoresis at 120 V for one hour in a buffer (Tris 3g/1, glycine 14.4 g/1, 0.1% SDS). The gel was removed, placed on a nitrocellulose filter, interposed between filter papers and electrophoresed at 5 V/cm, 4°C in a buffer (Tris 3g/1, glycine 14.4 g/1) to transferred proteins in the gel onto the nitrocellulose filter. The nitrocellulose filter was rinsed with TBS buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl), immersed into 400 ml of TBS buffer containing 3% gelatine and shaked at 40°C for one hour to block the nitrocellulose filter.

To TBS buffer containing 1% gelatine, there was added a monoclonal antibody directed to a non-A non-B hepatitis-specific antigen (OD_{280} = 4.3) with a dilution of 1/400. The resulting mixture and the nitrocellulose filter were put into a vinyl bag so that the mixture was present in an amount of 2 ml per filter. Reaction was effected at room temperature for 16 hours. The reaction mixture was washed three times with 400 ml of TBS buffer containing 0.05% Tween 20 for 10 minutes.

To TBS buffer containing 1% gelatine, there was added a labelled secondary antibody, anti-mouse IgG-PAP (horseradish peroxidase, Bio Rad), with a dilution of 1/1000. The resulting mixture and the nitrocellulose filter were put into a vinyl bag so that the mixture was present in an amount of 2 ml per filter. Reaction was effected at room temperature for 2 hours. The reaction mixture was washed three times with 400 ml of TBS buffer containing 0.05% Tween 20 for 10 minutes.

Color formation was effected by immersing the filter into 20 ml of TBS buffer containing 12 mg of 4-chloro-1-naphthol (Bio Rad) and hydrogen peroxide. After completion of color formation, the filter was thoroughly washed with water, put into a vinyl bag containing water, and stored in a dark and cold place.

Such a test effected showed that a protein reacting with

The monoclonal antibody was found at the same position (44 Kd) as found in the case of the specific antigen derived from infected chimpanzee liver. This verifies that such a specific antigen can be in fact expressed in E coli. The invention thus also relates to a process for the in vitro diagnosis of NON-A NON-B hepatitis, which comprises contacting a liver sample and/or a serum sample taken from a patient possibly infected with a NON-A NON-B hepatitis with the protein whose formula appears in claim 3 hereafter or a part thereof for a time and under conditions sufficient to allow for the production of a complex between said protein or part thereof with the antibodies contained in the patient sample and detecting the presence of the immunologic complex, particularly when the patient is suffering from NON-A NON-B hepatitis.

Any part of said protein,or any recombinant, produced by genetic engineering and including the aminoacid sequence of said protein or part of said protein can be substituted for above-said protein, it being understood that the said recombinant protein or part of said protein are specifically recognized by the same antibodies as those which recognize said protein.

In other words the invention relates to all recombinant proteins or protein fragments which bind to antibodies contained in a liver extract or serum sample, or both, and originating from a patient suffering from NON-A NON-B hepatitis.

The invention also relates to a process for detecting in vitro an infection by a NON-A NON-B hepatitis virus, which process comprises contacting the DNA of claim 4, or a fragment thereof, under suitable hybridization conditions, with a sample of liver extract and/or serum sample originating from the patient to be diagnosed and in which the nucleic acid components had previously been made accessible to hybridization, to form a hybridization product between said DNA of claim (probe) and the viral DNA of a NON-A NON-B hepatitis B virus, and detecting said hybridization product, particularly in the case where the patient is indeed infected with a NON-A NON-B virus.

Claims

1. A DNA fragment which contains a base sequence coding for a non-A non-B hepatitis-specific antigenic protein occurring in cells of the liver affected with non-A non-B hepatitis.

2. The DNA fragment in accordance with claim 1, in which the cells of the liver are derived from a human or chimpanzee individual.

3. The DNA fragment in accordance with claim 1, in which the antigenic protein specific to non-A non-B hepatitis has the whole or a part of the amino acid sequence represented by the formula:

```
                                                    10
Met Ala Val Thr Thr Arg Leu Thr Trp Leu His Glu Lys Ile Leu

                    20                                                  30
Gln Asn His Phe Gly Gly Lys Arg Leu Ser Leu Leu Tyr Lys Gly

                                                    40
Ser Val His Gly Phe His Asn Gly Val Leu Leu Asp Arg Cys Cys

                    50                                                  60
Asn Gln Gly Pro Thr Leu Thr Val Ile Tyr Ser Glu Asp His Ile

                                                    70
Ile Gly Ala Tyr Ala Glu Glu Gly Tyr Gln Glu Arg Lys Tyr Ala

                    80                                                  90
Ser Ile Ile Leu Phe Ala Leu Gln Glu Thr Lys Ile Ser Glu Trp

                                                   100
Lys Leu Gly Leu Tyr Thr Pro Glu Thr Leu Phe Cys Cys Asp Val

                   110                                                 120
Ala Lys Tyr Asn Ser Pro Thr Asn Phe Gln Ile Asp Gly Arg Asn
```

```
                                                130
Arg Lys Val Ile Met Asp Leu Lys Thr Met Glu Asn Leu Gly Leu

                    140                                     150
Ala Gln Asn Cys Thr Ile Ser Ile Gln Asp Tyr Glu Val Phe Arg

                                                160
Cys Glu Asp Ser Leu Asp Glu Arg Lys Ile Lys Gly Val Ile Glu

                    170                                     180
Leu Arg Lys Ser Leu Leu Ser Ala Leu Arg Thr Tyr Glu Pro Tyr

                                                190
Gly Ser Leu Val Gln Gln Ile Arg Ile Leu Leu Leu Gly Pro Ile

                    200                                     210
Gly Ala Gly Lys Ser Ser Phe Phe Asn Ser Val Arg Ser Val Phe

                                                220
Gln Gly His Val Thr His Gln Ala Leu Val Gly Thr Asn Thr Thr

                    230                                     240
Gly Ile Ser Glu Lys Tyr Arg Thr Tyr Ser Ile Arg Asp Gly Lys

                                                250
Asp Gly Lys Tyr Leu Pro Phe Ile Leu Cys Asp Ser Leu Gly Leu

                    260                                     270
Ser Glu Lys Glu Gly Gly Leu Cys Met Asp Asp Ile Ser Tyr Ile

                                                280
Leu Asn Gly Asn Ile Arg Asp Arg Tyr Gln Phe Asn Pro Met Glu

                    290                                     300
Ser Ile Lys Leu Asn His His Asp Tyr Ile Asp Ser Pro Ser Leu

                                                310
Lys Asp Arg Ile His Cys Val Ala Phe Val Phe Asp Ala Ser Ser

                    320                                     330
Ile Glu Tyr Phe Ser Ser Gln Met Ile Val Lys Ile Lys Arg Ile

                                                340
Arg Arg Glu Leu Val Asn Ala Gly Val Val His Val Ala Leu Leu

                    350                                     360
Thr His Val Asp Ser Met Asp Leu Ile Thr Lys Gly Asp Leu Ile

                                                370
Glu Ile Glu Arg Cys Val Pro Val Arg Ser Lys Leu Glu Glu Val

                    380                                     390
Gln Arg Lys Leu Gly Phe Ala Leu Ser Asp Ile Ser Val Val Ser
```

```
                                                  400
Asn Tyr Ser Ser Glu Trp Glu Leu Asp Pro Val Lys Asp Val Leu

                    410                                   420
Ile Leu Ser Ala Leu Arg Arg Met Leu Trp Ala Ala Asp Asp Phe

                                                  430
Leu Glu Asp Leu Pro Phe Glu Gln Ile Gly Asn Leu Arg Glu Glu

                    440
Ile Ile Asn Cys Ala Gln Gly Lys Lys ***.
```

4. The DNA fragment in accordance with claim 1, in which the base sequence comprises the whole or a part of the base sequence represented by the formula:

```
                10                  20                  30                  40
5' ATG GCA GTG ACA ACT CGT TTG ACA TGG TTG CAT GAA AAG ATC
3' --- --- --- --- --- --- --- --- --- --- --- --- --- ---

          50                  60                  70                  80
   CTG CAA AAT CAT TTT GGA GGG AAG CGG CTT AGC CTT CTC TAT
   --- --- --- --- --- --- --- --- --- --- --- --- --- ---

       90                 100                 110                 120
   AAG GGT AGT GTC CAT GGA TTC CAT AAT GGA GTT TTG CTT GAC
   --- --- --- --- --- --- --- --- --- --- --- --- --- ---

     130                 140                 150                 160
   AGA TGT TGT AAT CAA GGG CCT ACT CTA ACA GTG ATT TAT AGT
   --- --- --- --- --- --- --- --- --- --- --- --- --- ---

170                 180                 190                 200                 210
 GAA GAT CAT ATT ATT GGA GCA TAT GCA GAA GAG GGT TAC CAG
 --- --- --- --- --- --- --- --- --- --- --- --- --- ---

           220                 230                 240                 250
 GAA AGA AAG TAT GCT TCC ATC ATC CTT TTT GCA CTT CAA GAG
 --- --- --- --- --- --- --- --- --- --- --- --- --- ---

        260                 270                 280                 290
 ACT AAA ATT TCA GAA TGG AAA CTA GGA CTA TAT ACA CCA GAA
 --- --- --- --- --- --- --- --- --- --- --- --- --- ---

     300                 310                 320                 330
 ACA CTG TTT TGT TGT GAC GTT GCA AAA TAT AAC TCC CCA ACT
 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

```
  340           350           360           370
AAT TTC CAG ATA GAT GGA AGA AAT AGA AAA GTG ATT ATG GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

380           390           400           410           420
TTA AAG ACA ATG GAA AAT CTT GGA CTT GCT CAA AAT TGT ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

          430           440           450           460
ATC TCT ATT CAG GAT TAT GAA GTT TTT CGA TGC GAA GAT TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

        470           480           490           500
CTG GAC GAA AGA AAG ATA AAA GGG GTC ATT GAG CTC AGG AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

    510           520           530           540
AGC TTA CTG TCT GCC TTG AGA ACT TAT GAA CCA TAT GGA TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

  550           560           570           580
CTG GTT CAA CAA ATA CGA ATT CTG CTG CTG GGT CCA ATT GGA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

590           600           610           620           630
GCT GGG AAG TCT AGC TTT TTC AAC TCA GTG AGG TCT GTT TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

          640           650           660           670
CAA GGG CAT GTA ACG CAT CAG GCT TTG GTG GGC ACT AAT ACA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

        680           690           700           710
ACT GGG ATA TCT GAG AAG TAT AGG ACA TAC TCT ATT AGA GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

    720           730           740           750
GGG AAA GAT GGC AAA TAC CTG CCA TTT ATT CTG TGT GAC TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

  760           770           780           790
CTG GGG CTG AGT GAG AAA GAA GGC GGC CTG TGC ATG GAT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

800           810           820           830           840
ATA TCC TAC ATC TTG AAC GGT AAC ATT CGT GAT AGA TAC CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

          850           860           870           880
TTT AAT CCC ATG GAA TCA ATC AAA TTA AAT CAT CAT GAC TAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

```
          890             900             910             920
ATT GAT TCC CCA TCG CTG AAG GAC AGA ATT CAT TGT GTG GCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

     930             940             950             960
TTT GTA TTT GAT GCC AGC TCT ATT GAA TAC TTC TCC TCT CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

  970             980             990            1000
ATG ATA GTA AAG ATC AAA AGA ATT CGA AGG GAG TTG GTA AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

1010            1020            1030            1040            1050
GCT GGT GTG GTA CAT GTG GCT TTG CTC ACT CAT GTG GAT AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

          1060            1070            1080            1090
ATG GAT CTG ATT ACA AAA GGT GAC CTT ATA GAA ATA GAG AGA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

       1100            1110            1120            1130
TGT GTG CCT GTG AGG TCC AAG CTA GAG GAA GTC CAA AGA AAA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

    1140            1150            1160            1170
CTT GGA TTT GCT CTT TCT GAC ATC TCG GTG GTT AGC AAT TAT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

   1180            1190            1200            1210
TCC TCT GAG TGG GAG CTG GAC CCT GTA AAG GAT GTT CTA ATT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

1220            1230            1240            1250            1260
CTT TCT GCT CTG AGA CGA ATG CTA TGG GCT GCA GAT GAC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

          1270            1280            1290            1300
TTA GAG GAT TTG CCT TTT GAG CAA ATA GGG AAT CTA AGG GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

       1310            1320            1330
GAA ATT ATC AAC TGT GCA CAA GGA AAA AAA 3'
--- --- --- --- --- --- --- --- --- --- 5'
```

wherein the sign "-" represents a base complementary to the base shwon just above each sign.

5. An expression vector in which a DNA fragment containing a base sequence coding for a non-A non-B hepatitis-specific antigen is intoduced into a cloning site present downstream from a promoter of said vector.

6. The expression vector in accordance with claim 5, in which the promoter is controllable by a regulatory factor.

7. The expression vector in accordance with claim 5, in which the promoter operates in a microorganism.

8. The expression vector in accordance with claim 5, in which the promoter operates in an eukaryote.

9. A transformant obtained by transforming a host with an expression vector in which a DNA fragment containing a base sequence coding for a non-A non-B hepatitis-specific antigen is introduced into a cloning site present downstream from a promoter of said vector.

10. The transformant in accordance with claim 9, in which the host is Escherichia coli or Bacillus subtilis.

11. A process for producing an antigen specific to non-A non-B hepatitis comprising introducing a DNA fragment containing a base sequence coding for said specific antigen into a cloning site present downstream from a promoter of a vector for expression, introducing the expression vector containing said DNA fragment into a host, culturing said transformed host, and collecting the produced and accumulated antigen.

Fig. 1a

```
                    10                  20                  30
5' ATG GCA GTG ACA ACT CGT TTG ACA TGG TTG
3'

                    40                  50                  60
   CAT GAA AAG ATC CTG CAA AAT CAT TTT GGA

                    70                  80                  90
   GGG AAG CGG CTT AGC CTT CTC TAT AAG GGT

                   100                 110                 120
   AGT GTC CAT GGA TTC CAT AAT GGA GTT TTG

                   130                 140                 150
   CTT GAC AGA TGT TGT AAT CAA GGG CCT ACT

                   160                 170                 180
   CTA ACA GTG ATT TAT AGT GAA GAT CAT ATT

                   190                 200                 210
   ATT GGA GCA TAT GCA GAA GAG GGT TAC CAG

                   220                 230                 240
   GAA AGA AAG TAT GCT TCC ATC ATC CTT TTT

                   250                 260                 270
   GCA CTT CAA GAG ACT AAA ATT TCA GAA TGG

                   280                 290                 300
   AAA CTA GGA CTA TAT ACA CCA GAA ACA CTG
```

Fig. 1b

```
          310                 320                 330
TTT TGT TGT GAC GTT GCA AAA TAT AAC TCC

          340                 350                 360
CCA ACT AAT TTC CAG ATA GAT GGA AGA AAT

          370                 380                 390
AGA AAA GTG ATT ATG GAC TTA AAG ACA ATG

          400                 410                 420
GAA AAT CTT GGA CTT GCT CAA AAT TGT ACT

          430                 440                 450
ATC TCT ATT CAG GAT TAT GAA GTT TTT CGA

          460                 470                 480
TGC GAA GAT TCA CTG GAC GAA AGA AAG ATA

          490                 500                 510
AAA GGG GTC ATT GAG CTC AGG AAG AGC TTA

          520                 530                 540
CTG TCT GCC TTG AGA ACT TAT GAA CCA TAT

          550                 560                 570
GGA TCC CTG GTT CAA CAA ATA CGA ATT CTG

          580                 590                 600
CTG CTG GGT CCA ATT GGA GCT GGG AAG TCT
```

Fig. 1c

```
                610                 620                 630
AGC TTT TTC AAC TCA GTG AGG TCT GTT TTC

                640                 650                 660
CAA GGG CAT GTA ACG CAT CAG GCT TTG GTG

                670                 680                 690
GGC ACT AAT ACA ACT GGG ATA TCT GAG AAG

                700                 710                 720
TAT AGG ACA TAC TCT ATT AGA GAC GGG AAA

                730                 740                 750
GAT GGC AAA TAC CTG CCA TTT ATT CTG TGT

                760                 770                 780
GAC TCA CTG GGG CTG AGT GAG AAA GAA GGC

                790                 800                 810
GGC CTG TGC ATG GAT GAC ATA TCC TAC ATC

                820                 830                 840
TTG AAC GGT AAC ATT CGT GAT AGA TAC CAG

                850                 860                 870
TTT AAT CCC ATG GAA TCA ATC AAA TTA AAT

                880                 890                 900
CAT CAT GAC TAC ATT GAT TCC CCA TCG CTG
```

Fig. 1d

```
          910          920          930
AAG GAC AGA ATT CAT TGT GTG GCA TTT GTA

          940          950          960
TTT GAT GCC AGC TCT ATT GAA TAC TTC TCC

          970          980          990
TCT CAG ATG ATA GTA AAG ATC AAA AGA ATT

         1000         1010         1020
CGA AGG GAG TTG GTA AAC GCT GGT GTG GTA

         1030         1040         1050
CAT GTG GCT TTG CTC ACT CAT GTG GAT AGC

         1060         1070         1080
ATG GAT CTG ATT ACA AAA GGT GAC CTT ATA

         1090         1100         1110
GAA ATA GAG AGA TGT GTG CCT GTG AGG TCC

         1120         1130         1140
AAG CTA GAG GAA GTC CAA AGA AAA CTT GGA

         1150         1160         1170
TTT GCT CTT TCT GAC ATC TCG GTG GTT AGC

         1180         1190         1200
AAT TAT TCC TCT GAG TGG GAG CTG GAC CCT
```

Fig. 2

AAAAATTTATTTGCTTTCAGGAAAATTTTTCTGT
TTTTTAAATAAACGAAAGTCCTTTTAAAAAGACA

ATAATGTGTGGAATTGTGAGCGGATAACAATTTC
TATTACACACCTTAACACTCGCCTATTGTTAAAG

Fig. 1e

```
          1210          1220          1230
GTA AAG GAT GTT CTA ATT CTT TCT GCT CTG

          1240          1250          1260
AGA CGA ATG CTA TGG GCT GCA GAT GAC TTC

          1270          1280          1290
TTA GAG GAT TTG CCT TTT GAG CAA ATA GGG

          1300          1310          1320
AAT CTA AGG GAG GAA ATT ATC AAC TGT GCA

          1330
CAA GGA AAA AAA 3'
                5'
```

Fig. 3a

```
          250          260          270          280          290          300
CTA GGA CTA TAT ACA CCA GAA ACA CTG TTT TGT TGT GAC GTT GCA AAA TAT AAC TCC CCA
Leu Gly Leu Tyr Thr Pro Glu Thr Leu Phe Cys Cys Asp Val Ala Lys Tyr Asn Ser Pro

          310          320          330          340          350          360
ACT AAT TTC CAG ATA GAT GGA AGA AAT AGA AAA GTG ATT ATG GAC TTA AAG ACA ATG GAA
Thr Asn Phe Gln Ile Asp Gly Arg Asn Arg Lys Val Ile Met Asp Leu Lys Thr Met Glu

          370          380          390          400          410          420
AAT CTT GGA CTT GCT CAA AAT TGT ACT ATC TCT ATT CAG GAT TAT GAA GTT TTT CGA TGC
Asn Leu Gly Leu Ala Gln Asn Cys Thr Ile Ser Ile Gln Asp Tyr Glu Val Phe Arg Cys

          430          440          450          460          470          480
GAA GAT TCA CTG GAC GAA AGA AAG ATA AAA GGG GTC ATT GAG CTC AGG AAG AGC TTA CTG
Glu Asp Ser Leu Asp Glu Arg Lys Ile Lys Gly Val Ile Glu Leu Arg Lys Ser Leu Leu

          490          500          510          520          530          540
TCT GCC TTG AGA ACT TAT GAA CCA TAT GGA TCC CTG GTT CAA CAA ATA CGA ATT CTG CTG
Ser Ala Leu Arg Thr Tyr Glu Pro Tyr Gly Ser Leu Val Gln Gln Ile Arg Ile Leu Leu

          550          560          570          580          590          600
CTG GGT CCA ATT GGA GCT GGG AAG TCT AGC TTT TTC AAC TCA GTG AGG TCT GTT TTC CAA
Leu Gly Pro Ile Gly Ala Gly Lys Ser Ser Phe Phe Asn Ser Val Arg Ser Val Phe Gln
```

0293274

Fig. 3b

```
          610         620         630         640         650         660
GGG CAT GTA ACG CAT CAG GCT TTG GTG GGC ACT AAT ACA ACT GGG ATA TCT GAG AAG TAT
Gly His Val Thr His Gln Ala Leu Val Gly Thr Asn Thr Thr Gly Ile Ser Glu Lys Tyr

          670         680         690         700         710         720
AGG ACA TAC TCT ATT AGA GAC GGG AAA GAT GGC AAA TAC CTG CCA TTT ATT CTG TGT GAC
Arg Thr Tyr Ser Ile Arg Asp Gly Lys Asp Gly Lys Tyr Leu Pro Phe Ile Leu Cys Asp

          730         740         750         760         770         780
TCA CTG GGG CTG AGT GAG AAA GAA GGC GGC CTG TGC ATG GAT GAC ATA TCC TAC ATC TTG
Ser Leu Gly Leu Ser Glu Lys Glu Gly Gly Leu Cys Met Asp Asp Ile Ser Tyr Ile Leu

          790         800         810         820         830         840
AAC GGT AAC ATT CGT GAT AGA TAC CAG TTT AAT CCC ATG GAA TCA ATC AAA TTA AAT CAT
Asn Gly Asn Ile Arg Asp Arg Tyr Gln Phe Asn Pro Met Glu Ser Ile Lys Leu Asn His

          850         860         870         880         890         900
CAT GAC TAC ATT GAT TCC CCA TCG CTG AAG GAC AGA ATT CAT TGT GTG GCA TTT GTA TTT
His Asp Tyr Ile Asp Ser Pro Ser Leu Lys Asp Arg Ile His Cys Val Ala Phe Val Phe

          910         920         930         940         950         960
GAT GCC AGC TCT ATT GAA TAC TTC TCC TCT CAG ATG ATA GTA AAG ATC AAA AGA ATT CGA
Asp Ala Ser Ser Ile Glu Tyr Phe Ser Ser Gln Met Ile Val Lys Ile Lys Arg Ile Arg

          970         980         990         1000        1010        1020
AGG GAG TTG GTA AAC GCT GGT GTG GTA CAT GTG GCT TTG CTC ACT CAT GTG GAT AGC ATG
Arg Glu Leu Val Asn Ala Gly Val Val His Val Ala Leu Leu Thr His Val Asp Ser Met
```

Fig. 3c

```
          1030          1040          1050          1060          1070          1080
GAT CTG ATT ACA AAA GGT GAC CTT ATA GAA ATA GAG AGA TGT GTG CCT GTG AGG TCC AAG
Asp Leu Ile Thr Lys Gly Asp Leu Ile Glu Ile Glu Arg Cys Val Pro Val Arg Ser Lys

          1090          1100          1110          1120          1130          1140
CTA GAG GAA GTC CAA AGA AAA CTT GGA TTT GCT CTT TCT GAC ATC TCG GTG GTT AGC AAT
Leu Glu Glu Val Gln Arg Lys Leu Gly Phe Ala Leu Ser Asp Ile Ser Val Val Ser Asn

          1150          1160          1170          1180          1190          1200
TAT TCC TCT GAG TGG GAG CTG GAC CCT GTA AAG GAT GTT CTA ATT CTT TCT GCT CTG AGA
Tyr Ser Ser Glu Trp Glu Leu Asp Pro Val Lys Asp Val Leu Ile Leu Ser Ala Leu Arg

          1210          1220          1230          1240          1250          1260
CGA ATG CTA TGG GCT GCA GAT GAC TTC TTA GAG GAT TTG CCT TTT GAG CAA ATA GGG AAT
Arg Met Leu Trp Ala Ala Asp Asp Phe Leu Glu Asp Leu Pro Phe Glu Gln Ile Gly Asn

          1270          1280          1290          1300
CTA AGG GAG GAA ATT ATC AAC TGT GCA CAA GGA AAA AAA TAG
Leu Arg Glu Glu Ile Ile Asn Cys Ala Gln Gly Lys Lys ***
```

Fig. 4a

```
          10         20         30         40         50         60         70         80
5' GGGGGGCTAC CCTCAGCTCT AGCTCATACT ACAGACAGTA CAACAGATCA AGAAGTATGG CAGTGACAAC TCGTTTGACA
3' CCCCCCGATG GGAGTCGAGA TCGAGTATGA TGTCTGTCAT GTTGTCTAGT TCTTCATACC GTCACTGTTG AGCAAACTGT

          90        100        110        120        130        140        150        160
   TGGTTGCATG AAAAGATCCT GCAAAATCAT TTTGGAGGGA AGCGGCTTAG CCTTCTCTAT AAGGGTAGTG TCCATGGATT
   ACCAACGTAC TTTTCTAGGA CGTTTTAGTA AAACCTCCCT TCGCCGAATC GGAAGAGATA TTCCCATCAC AGGTACCTAA

         170        180        190        200        210        220        230        240
   CCATAATGGA GTTTTGCTTG ACAGATGTTG TAATCAAGGG CCTACTCTAA CAGTGATTTA TAGTGAAGAT CATATTATTG
   GGTATTACCT CAAAACGAAC TGTCTACAAC ATTAGTTCCC GGATGAGATT GTCACTAAAT ATCACTTCTA GTATAATAAC

         250        260        270        280        290        300        310        320
   GAGCATATGC AGAAGAGGGT TACCAGGAAA GAAAGTATGC TTCCATCATC CTTTTTGCAC TTCAAGAGAC TAAAATTTCA
   CTCGTATACG TCTTCTCCCA ATGGTCCTTT CTTTCATACG AAGGTAGTAG GAAAAACGTG AAGTTCTCTG ATTTTAAAGT

         330        340        350        360        370        380        390        400
   GAATGGAAAC TAGGACTATA TACACCAGAA ACACTGTTTT GTTGTGACGT TGCAAAATAT AACTCCCCAA CTAATTTCCA
   CTTACCTTTG ATCCTGATAT ATGTGGTCTT TGTGACAAAA CAACACTGCA ACGTTTTATA TTGAGGGGTT GATTAAAGGT

         410        420        430        440        450        460        470        480
   GATAGATGGA AGAAATAGAA AAGTGATTAT GGACTTAAAG ACAATGGAAA ATCTTGGACT TGCTCAAAAT TGTACTATCT
   CTATCTACCT TCTTTATCTT TTCACTAATA CCTGAATTTC TGTTACCTTT TAGAACCTGA ACGAGTTTTA ACATGATAGA

         490        500        510        520        530        540        550        560
   CTATTCAGGA TTATGAAGTT TTTCGATGCG AAGATTCACT GGACGAAAGA AAGATAAAAG GGGTCATTGA GCTCAGGAAG
   GATAAGTCCT AATACTTCAA AAAGCTACGC TTCTAAGTGA CCTGCTTTCT TTCTATTTTC CCCAGTAACT CGAGTCCTTC
```

0293274

Fig. 4b

```
       570        580        590        600        610        620        630        640
AGCTTACTGT CTGCCTTGAG AACTTATGAA CCATATGGAT CCCTGGTTCA ACAAATACGA ATTCTGCTGC TGGGTCCAAT
TCGAATGACA GACGGAACTC TTGAATACTT GGTATACCTA GGGACCAAGT TGTTTATGCT TAAGACGACG ACCCAGGTTA

       650        660        670        680        690        700        710        720
TGGAGCTGGG AAGTCTAGCT TTTTCAACTC AGTGAGGTCT GTTTTCCAAG GGCATGTAAC GCATCAGGCT TTGGTGGGCA
ACCTCGACCC TTCAGATCGA AAAAGTTGAG TCACTCCAGA CAAAAGGTTC CCGTACATTG CGTAGTCCGA AACCACCCGT

       730        740        750        760        770        780        790        800
CTAATACAAC TGGGATATCT GAGAAGTATA GGACATACTC TATTAGAGAC GGGAAAGATG GCAAATACCT GCCATTTATT
GATTATGTTG ACCCTATAGA CTCTTCATAT CCTGTATGAG ATAATCTCTG CCCTTTCTAC CGTTTATGGA CGGTAAATAA

       810        820        830        840        850        860        870        880
CTGTGTGACT CACTGGGGCT GAGTGAGAAA GAAGGCGGCC TGTGCATGGA TGACATATCC TACATCTTGA ACGGTAACAT
GACACACTGA GTGACCCCGA CTCACTCTTT CTTCCGCCGG ACACGTACCT ACTGTATAGG ATGTAGAACT TGCCATTGTA

       890        900        910        920        930        940        950        960
TCGTGATAGA TACCAGTTTA ATCCCATGGA ATCAATCAAA TTAAATCATC ATGACTACAT TGATTCCCCA TCGCTGAAGG
AGCACTATCT ATGGTCAAAT TAGGGTACCT TAGTTAGTTT AATTTAGTAG TACTGATGTA ACTAAGGGGT AGCGACTTCC

       970        980        990       1000       1010       1020       1030       1040
ACAGAATTCA TTGTGTGGCA TTTGTATTTG ATGCCAGCTC TATTGAATAC TTCTCCTCTC AGATGATAGT AAAGATCAAA
TGTCTTAAGT AACACACCGT AAACATAAAC TACGGTCGAG ATAACTTATG AAGAGGAGAG TCTACTATCA TTTCTAGTTT

       1050       1060       1070       1080       1090       1100       1110       1120
AGAATTCGAA GGGAGTTGGT AAACGCTGGT GTGGTACATG TGGCTTTGCT CACTCATGTG GATAGCATGG ATCTGATTAC
TCTTAAGCTT CCCTCAACCA TTTGCGACCA CACCATGTAC ACCGAAACGA GTGAGTACAC CTATCGTACC TAGACTAATG
```

Fig. 4c

```
      1130        1140        1150        1160        1170        1180        1190        1200
AAAAGGTGAC CTTATAGAAA TAGAGAGATG TGTGCCTGTG AGGTCCAAGC TAGAGGAAGT CCAAAGAAAA CTTGGATTTG
TTTTCCACTG GAATATCTTT ATCTCTCTAC ACACGGACAC TCCAGGTTCG ATCTCCTTCA GGTTTCTTTT GAACCTAAAC

      1210        1220        1230        1240        1250        1260        1270        1280
CTCTTTCTGA CATCTCGGTG GTTAGCAATT ATTCCTCTGA GTGGGAGCTG GACCCTGTAA AGGATGTTCT AATTCTTTCT
GAGAAAGACT GTAGAGCCAC CAATCGTTAA TAAGGAGACT CACCCTCGAC CTGGGACATT TCCTACAAGA TTAAGAAAGA

      1290        1300        1310        1320        1330        1340        1350        1360
GCTCTGAGAC GAATGCTATG GGCTGCAGAT GACTTCTTAG AGGATTTGCC TTTTGAGCAA ATAGGGAATC TAAGGGAGGA
CGAGACTCTG CTTACGATAC CCGACGTCTA CTGAAGAATC TCCTAAACGG AAAACTCGTT TATCCCTTAG ATTCCCTCCT

      1370        1380        1390        1400        1410        1420        1430        1440
AATTATCAAC TGTGCACAAG GAAAAAAATA GATATGTGAA AGGTTCACGT AAATTTCCTC ACATCACAGA AGATTAAAAT
TTAATAGTTG ACACGTGTTC CTTTTTTTAT CTATACACTT TCCAAGTGCA TTTAAAGGAG TGTAGTGTCT TCTAATTTTA

      1450        1460        1470        1480        1490        1500        1510        1520
TCAGAAAGGA GAAAACACAG ACCAAAGAGA AGTAACTAAG ACCAAAGGGA TGTGTTTTAT TAATGTCTAG GATGAAGAAA
AGTCTTTCCT CTTTTGTGTC TGGTTTCTCT TCATTGATTC TGGTTTCCCT ACACAAAATA ATTACAGATC CTACTTCTTT

      1530        1540        1550        1560        1570        1580        1590        1600
TGCATAGAAC ATTGTAGTAC TTGTAAATAA CTAGAAATAA CATGATTTAG TCATAATTGT GAAAAATAAT AATAATTTTT
ACGTATCTTG TAACATCATG AACATTTATT GATCTTTATT GTACTAAATC AGTATTAACA CTTTTTATTA TTATTAAAAA

      1610        1620        1630        1640        1650        1660
CTTGGATTTA TGTTCTGTAT CTGTGAAAAA ATAAATTTCT TATAAAAAAA AAAAAAAAAA AAAAAAA 3'
GAACCTAAAT ACAAGACATA GACACTTTTT TATTTAAAGA ATATTTTTTT TTTTTTTTTT TTTTTTT 5'
```

Fig. 5

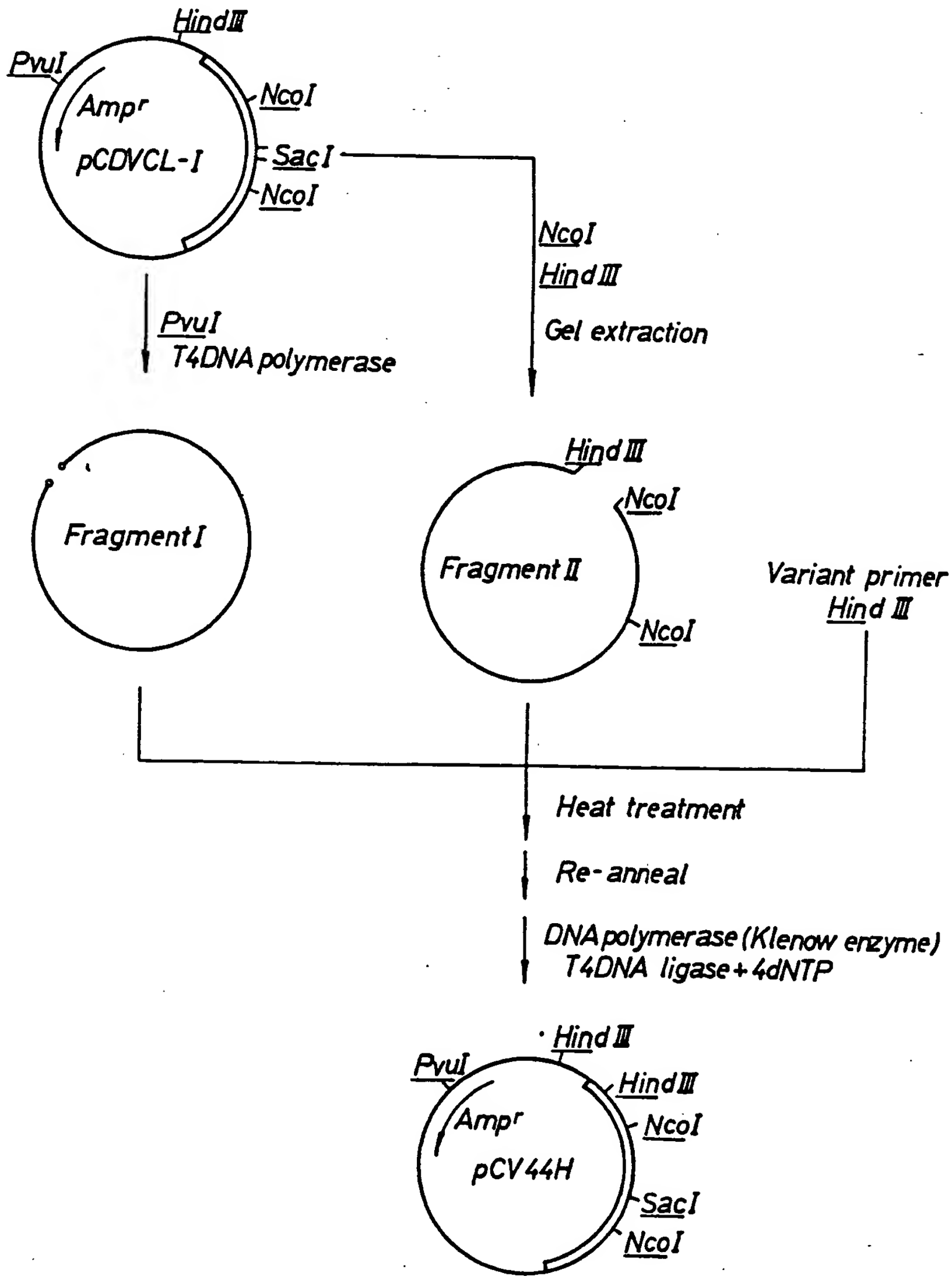
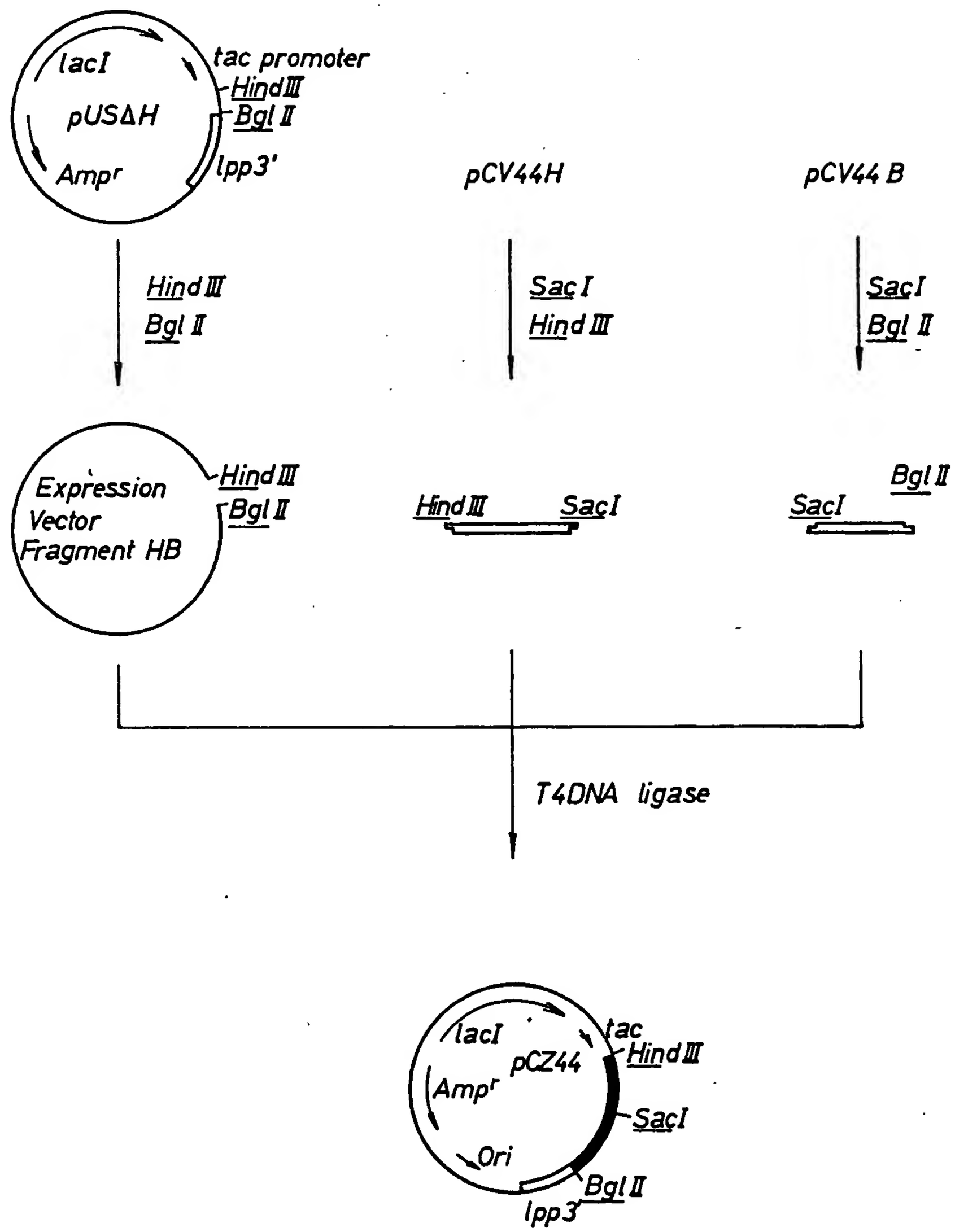

HindIII
PvuI
NcoI
Ampr
pCDVCL-I
SacI
NcoI
NcoI
HindIII
Gel extraction
PvuI
T4DNA polymerase
Fragment I
HindIII
NcoI
Fragment II
NcoI
Variant primer
HindIII
Heat treatment
Re-anneal
DNA polymerase (Klenow enzyme)
T4DNA ligase + 4dNTP
HindIII
PvuI
HindIII
Ampr
NcoI
pCV44H
SacI
NcoI

Fig.6

PvuI
Ampr
pCDVCL-I
NcoI
SacI
NcoI
NsiI
PvuI
T4 DNA polymerase
NcoI
NsiI
Gel extraction
Fragment I
Fragment II
NcoI
NcoI
NsiI
Variant primer
BglII
Heat treatment
Re-anneal
DNA polymerase(Klenow enzyme)
T4 DNA ligase
+4dNTP
PvuI
Ampr
pCV44B
NcoI
SacI
NcoI
BglII
NsiI

Fig.7


lacI
tac promoter
HindIII
BglII
pUSΔH
Ampr
lpp3'
pCV44H
pCV44B
HindIII
BglII
SacI
HindIII
SacI
Expression Vector Fragment HB
HindIII
BglII
HindIII
SacI
SacI
BglII
T4DNA ligase
lacI
tac
HindIII
pCZ44
Ampr
SacI
Ori
BglII
lpp3'

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 40 0790

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,Y	EP-A-0 190 972 (MITSUBISHI) * Whole document * ---	1-11	C 12 N 15/00 A 61 K 39/29
D,Y	PROC. NATL. ACAD. SCI. USA, vol. 80, March 1983, pages 1194-1198; R.A. YOUNG et al.: "Efficient isolation of genes by using antibody probes" * Whole article * ---	1-11	
A	CHEMICAL ABSTRACTS, vol. 105, no. 17, October 1986, page 527, no. 151110y, Columbus, Ohio, US; T. AKATSUKA et al.: "Non-A, non-B hepatitis related AN6520 Ag is a normal cellular protein mainly expressed in liver II" & J. MED. VIROL. 1986, 20(1), 43-56 * Whole abstract * ---	1	
A	BIOLOGICAL ABSTRACTS, vol. 80, 1985, no. 4871; J.-I. TOHMATSU et al.: "AN6520 antigen: an antigen purified from liver with non-A, non-B hepatitis" & J. MED. VIROL 15(4): 357-372. 1985. * Whole abstract * ---	1	
A	EP-A-0 066 296 (EISAI CO., LTD) ---		TECHNICAL FIELDS SEARCHED (Int. Cl.4)
A	EP-A-0 092 249 (EISAI CO., LTD) -----		C 12 N A 61 K G 01 N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21-06-1988	SKELLY J.M.

EPO FORM 1503 03.82 (P0401)

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 293 274 B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication of patent specification: 04.09.91 Bulletin 91/36

(51) Int. Cl.[5]: C12N 15/12, C12N 15/33

(21) Application number: 88400790.7

(22) Date of filing: 31.03.88

(54) DNA molecules encoding non-A, non-B hepatitis antigens, and their use in producing said antigens.

(30) Priority: 31.03.87 JP 78313/87
04.06.87 JP 140586/87
10.11.87 JP 283990/87

(43) Date of publication of application: 30.11.88 Bulletin 88/48

(45) Publication of the grant of the patent: 04.09.91 Bulletin 91/36

(84) Designated Contracting States: CH DE FR GB IT LI NL SE

(56) References cited:
EP-A- 0 066 296
EP-A- 0 092 249
EP-A- 0 190 972
PROC. NATL. ACAD. SCI. USA, vol. 80, March 1983, pages 1194-1198; R.A. YOUNG et al.: "Efficient isolation of genes by using antibody probes"
CHEMICAL ABSTRACTS, vol. 105, no. 17, October 1986, page 527, no. 151110y, Columbus, Ohio, US; T. AKATSUKA et al.: "Non-A, non-B hepatitis related AN6520 Ag is a normal cellular protein mainly expressed in liver II" & J. MED. VIROL. 1986, 20(1), 43-56
BIOLOGICAL ABSTRACTS, vol. 80, 1985, no. 4871; J.-I. TOHMATSU et al.: "AN6520 antigen: an antigen purified from liver with non-A, non-B hepatitis" & J. MED. VIROL. 15(4): 357-372. 1985.

(73) Proprietor: MITSUBISHI KASEI CORPORATION
5-2, Marunouchi 2-chome Chiyoda-ku
Tokyo 100 (JP)

(72) Inventor: Takahashi, Kazuhiro 303 Corpo Omodaka
29 Ichijoji-Somedono-cho Sakyo-ku
Kyoto City Kyoto Prefecture (JP)
Inventor: Shibui, Tatsurou
M606-11 San White 6-15-4 Minami-Naruse
Machida City Tokyo (JP)
Inventor: Kamizono, Michiru
F202-24 San White 2-8-11 Tsuruma
Machida City Tokyo (JP)
Inventor: Matsui, Rie
A211-23 San White 1-26-31 Ogawa
Machida City Tokyo (JP)
Inventor: Teranishi, Yutaka
1-14-2 Takane
Sagamihara City Kanagawa Prefecture (JP)
Inventor: Nakanishi, Shigetada
517-116 Iwakura-Nagatani-cho Sakyo-ku
Kyoto City Kyoto Prefecture (JP)
Inventor: Kitamura, Naomi
2-272 Yakumo-Higashi-machi
Moriguchi City Osaka Prefecture (JP)

(74) Representative: Gutmann, Ernest et al
S.C. Ernest Gutmann - Yves Plasseraud 67, boulevard Haussmann
F-75008 Paris (FR)

EP 0 293 274 B1

Description

The present invention generally relates to the production of an antigen specific to non-A non-B hepatitis by recombinant DNA technology. More particularly, it relates to a DNA fragment coding for an antigen specifically occurring in a host affected with non-A non-B hepatitis, an expression vector containing such a DNA fragment, a host transformed with such an expression vector, as well as a process for producing said antigen specific to non-A non-B hepatitis by culturing such a transformant.

Among viral hepatitises, the viral entities of hepatitis type A and type B have been found and, accordingly, it has now become possible to diagnose such diseases by immunological methods.

Still another type of hepatitis different from the types A and B, which is called non-A non-B type hepatitis, is said to be over 90% of post-transfusion hepatitis : refer to NIPPON RINSHO (Japan Clinic), 35, 2724 (1977); J. Biol. Med., 49, 243 (1976). The pathogenic virus of the non-A non-B type hepatitis, however, has not yet been identified. Only one fact which has already been established is potential infection of human hepatitis type non-A non-B virus to chimpanzee : refer to Lancet I, 459 (1978) ; Ibid., 463 (1978).

Many workers have done various investigations for searching an antigen-antibody system related to the non-A non-B hepatitis by using mainly sera from patients affected with the disease ; nevertheless, no definite system has been found. Under these circumstances, the diagnosis of non-A non-B hepatitis should inevitably be effected by so-called exclusion diagnosis : that is, whether or not the hepatitis of a patient is type A or type B or other hepatitis due to a virus known to cause hepatopathy, for example, CMV, HSV, EBV, etc., is first determined ; and if not, the patient's hepatitis is diagnosed as non-A non-B type. Thus, such a diagnosis of non-A non-B hepatitis will require much time and labor.

An antigenic protein specific to non-A non-B hepatitis and useful for the direct diagnoses of the hepatitis has been purified from human and chimpanzee hepatocytes affected with non-A non-B hepatitis, and a monoclonal antibody specific to the antigen and useful for the treatment of the non-A non-B hepatitis has also been proposed : refer to Japanese Patent Application Laying-open (KOKAI) Nos. 176856/86 and 56196/86.

A large amount of such an antigenic protein specific to non-A non-B hepatitis should be required when such a protein is to be employed, for example, as a diagnostic agent. However, it is not always appropriate to purify such a large amount of the antigenic protein from chimpanzee hepatocytes affected with non-A non-B hepatitis.

On the other hand, in order to detect a gene coding for a specific antigen of non-A non-B hepatitis by nucleic acid hybridization and, further, to produce such an antigen specific to non-A non-B hepatitis by the recombinant DNA technology, it is essential to obtain a gene fragment coding for the antigenic protein specific to the non-A non-B hepatitis.

The present inventors have made great efforts to produce such a specific antigenic protein in a large amount by genetic engineering techniques, and finally isolated a gene fragment coding for the antigenic protein specific to non-A non-B hepatitis, said gene fragment being useful for the production of such antigens. Further, the inventors have successfully constructed an expression vector containing said gene fragment. Thus, the present invention has now been attained.

It is an object of the invention to provide a DNA fragment which contains a base sequence coding for an antigen specifically occurring in a host cell affected with non-A non-B hepatitis or an antigenic protein specific to non-A non-B hepatitis having physiological activities equivalent to those of said specifically occurring antigen.

Another object of the invention is to provide an expression vector having said DNA fragment introduced thereinto at a cloning site downstream from a promoter of the vector.

A still another object of the invention is to provide a transformant obtained by transforming a host cell with said expression vector.

A further object of the invention is to provide a process for producing such an antigen specific to non-A non-B hepatitis by culturing said transformant.

Other objects and advantages of the present invention will be apparent from the following detailed description with reference to the attached drawings, in which :

Figs. 1a-1e show the base sequence coding for an antigenic protein specific to non-A non-B hepatitis ;

Fig. 2 shows the base sequence of a hybrid promoter Pac ;

Figs. 3a-3c show the base sequence of a cDNA fragment obtained in Example 1 described hereinbelow, together with deduced amino acid sequence ;

Figs. 4a-4c show the base sequence of cDNA containing the full length gene sequence of an antigenic protein specific to non-A non-B hepatitis, which cDNA was obtained in Example 2 described hereinbelow, the base sequence 57-1388 thereof coding for the antigenic protein specific to non-A non-B hepatitis ;

Fig. 5 schematically illustrates the construction of a plasmid pCV44H ;

Fig. 6 schematically illustrates the construction of a plasmid pCV44B ; and

Fig. 7 schematically illustrates the construction of a plasmid pCZ44.

The present invention will be described in detail hereinbelow.

According to one aspect of the invention, a DNA fragment is provided which contains a base sequence coding for an antigenic protein occurring specifically in hepatocytes affected with non-A non-B hepatitis.

Such a DNA fragment of the invention may be prepared in the following manner.

First, a liver tissue specimen derived from a human or chimpanzee individual affected with non-A non-B hepatitis is homogenized in an aqueous solution of guanidinium thiocyanate and then subjected to cesium chloride equilibrium density gradient centrifugation according to Chirgwin et al. method (Biochemistry, 18, 5294-5299 (1979)) to separate total RNA as a precipitate. After separation, the total RNA is purified by phenol extraction and ethanol precipitation.

"Individuals affected with non-A non-B hepatitis" used as sources of liver tissue specimens in the invention may include those affected with so-called type D hepatitis, which has recently been named.

It is known that mRNA of an antigen gene generally has a poly-A chain. Thus, the total RNA is subjected to oligo(dT) cellulose column chromatography in a conventional manner and poly(A)-containing RNA (poly A^+ RNA) is isolated as mRNA material.

A cDNA library corresponding to the poly A^+ RNA is then obtained from the mRNA material according to the random primer method (Y. Ebina et al., Cell, 40, 747-758 (1980)) : Thus, a number of DNAs complementary to the mRNA material are randomly synthesized using any primer of e.g. about 6 bases and a reverse transcriptase.

The cDNA is methylated with a DNA methylase, e.g. EcoRI methylase, to protect cleavage sites present in the cDNA capable of being cleaved by a corresponding restriction enzyme, e.g. EcoRI. A DNA linker containing the corresponding restriction enzyme cleavage sites at both ends, e.g. EcoRI linker (CGAATTCG), is added to the methylated cDNA and, then, this cDNA is digested with the restriction enzyme, e.g. EcoRI.

The digested cDNA is then cloned into a cloning vector such as a plasmid or a λ phage. For example, the cDNA may be introduced into EcoRI site of λgt 11 DNA, which is an expression cloning vector : refer to R.A. Young et al., Pro. Natl. Acad. Scl. U.S.A., 80, 1194-1198 (1983). The cDNA will be inserted into the β-gal gene on the λgt 11 phage. Thus, expression of the cDNA can be easily verified by the production of a fused protein with β-galactosidase due to induction of the expression by the lactose operon promoter of said phage when E. coli transfected with said phage is cultured in a medium containing IPTG (isopropylthio-β-D-galactopyranoside).

The λgt 11 phage incorporating the cDNA is then introduced into E. coli by Tomizawa et al. method in "Experimental Procedures for Bacteriophages", pp. 99-174, published May 30, 1970 by Iwanami Shoten (Japan). The thus transfected microorganism is cultured in an IPTG-containing medium.

The thus formed plaques can be easily selected by an immunological screening method using a monoclonal antibody specifically directed to non-A non-B hepatitis to obtain a desired cDNA. Such a monoclonal antibody which can be used in the immunological screening method may be prepared according to the methods described in Japanese Patent Application Laying-open Nos. 56196/86 and 91328/88. The screening methods used may include the western blotting technique described in these applications.

The plaques positive in the immunological screening test are selected to proliferate the phage by Tomizawa et al. method. DNA is purified from the grown phage by T. Maniatis et al. method in "Molecular Cloning", Cold Spring Harbor Laboratory, pp. 85 et seq. (1982), and cleaved with a suitable restriction enzyme such as EcoRI. The thus purified and digested DNA fragments can be used to determine the base sequence of a desired cDNA segment according to Maxam and Gilbert method in Methods in Enzymology, 65, 499-560 (1980) ; or alternatively, after further cloning the DNA fragments into M13 phage, the base sequence of such a desired cDNA segment can be determined according to the dideoxy method : Sanger et al., Proc. Natl. Acad. Sci. U.S.A., 74, 5463 (1977).

Thus, a cDNA fragment coding for an antigen specific to non-A non-B hepatitis can be obtained. However, such a DNA fragment may usually be only a portion of the gene coding for the non-A non-B hepatitis-specific antigen.

A full length cDNA coding for such a non-A non-B hepatitis-specific antigen may be obtained in the following manner.

Poly A^+-mRNA is isolated and purified in a manner similar to that described above. From the poly A^+-mRNA a cDNA library is obtained according to Okayama-Berg vector-primer method : Molecular and Cellular Biology, 2, 161-170 (1982).

A plasmid containing such a cDNA thus prepared is used to transform E. coli by any conventional method, for instance, the method D. Hanahan : J. Mol. Biol., 166, 557 (1983). The transformant ampicillin-resistant strains are collected and screened by the colony hybridization method using the aforementioned DNA fragment as a probe. Such a probe may preferably be prepared by either the strepto-avidin method, or the nick translation method using photobiotinnucleic acids and ^{32}P-nucleic acids.

The thus selected colonies containing a cDNA clone are cultured. Plasmid DNA is obtained from the cul-

tured colony according to Birnboim et al. method (Nucleic Acid Res., 7, 1513 (1979)) and digested with a suitable restriction enzyme. The base sequence of a desired full-length cDNA segment is then determined according to the aforementioned Maxam and Gilbert method or, alternatively, after further cloning the digested DNA into M13 phage or pVC12 plasmid, such a base sequence is determined according to the above described Sanger et al. dideoxy method.

The base sequence of the full length DNA coding for an antigen specific to non-A non-B hepatitis is shown in Fig. 1, in which the symbol "–" just under the base sequence represents a corresponding base complementary to the respective base described just above each of the symbols.

Of course, DNA fragments which can be employed in the invention do not necessarily contain the same base sequence as shown in Fig. 1, but those DNA fragments in which a part of said base sequence shown in Fig. 1 has been substituted by at least one different base or deleted therefrom and those DNA fragments in which one or more additional bases have been added to the base sequence of Fig. 1 may also be included herein provided that such different DNA fragments may code for substances having physiological activities equivalent to those of the non-A non-B hepatitis-specific antigens encoded by the base sequence of Fig. 1.

According to another aspect of the invention, an expression vector is provided in which the aforementioned DNA fragment of the invention is inserted into a cloning site downstream from a promoter of this vector.

The expression vector of the invention contains a promoter in a position capable of controlling the transcription of a DNA fragment coding for a non-A non-B hepatitis-specific antigen obtained by the aforementioned method. The promoters used in the invention may be any promoter capable of expressing the DNA fragment in a host, and preferably of controlling the transcription of the fragment.

When a host used is a microorganism such as Escherichia coli, Bacillus subtilis, etc., the expression vector of the invention may preferably comprise a promoter, a ribosome binding sequence, a gene for a non-A non-B hepatitis-specific antigen, a transcription termination factor, and a gene controlling the promoter.

The promoter used may include those derived from E. coli, phage, etc., for example, tryptophan synthase operon (trp), lactose operon (lac), lipoprotein (lpp), recA, lambda phage P_L, P_R, T5 early gene P_{25}, P_{26} promoter, which may also be prepared by chemical synthesis. Also included herein are hybrid promoters such as tac (trp: lac), trc (trp : lac) and Pac (phage : E. coli) shown in Fig. 2.

The ribosome binding sequence may be derived from E. coli, phage, etc., but preferably may be those synthetically prepared, for example, those containing a consensus sequence such as

AGGAGGTTTAA.
SD sequence

The gene for a non-A non-B hepatitis-specific antigen may be directly employed without any modification. Preferably, an unnecessary base sequence (non-coding region) may be deleted by site-directed mutagenesis: BIO TECHNOLOGY, July, 636-639 (1984).

A transcription termination factor may not always be required in the expression vector of the invention. Preferably, the instant vector may contain a ρ-independent terminator, for example, lpp terminator, trp operon terminator, ribosomal RNA gene terminator, etc.

The expression vector may be derived from any conventional plasmid. Preferably, it may be derived from such a plasmid as replicating itself in E. coli or Bacillus subtilis, for example, pBR322- or pUB110-derived plasmid.

Desirably, these factors required for expression are arranged in the expression plasmid in the order of the promoter, the SD sequence, the structural gene of a non-A non-B hepatitis-specific antigen, and the transcription termination factor from 5′ to 3′. A repressor gene required to control the transcription, a marker gene such as drug-resistant gene, and a plasmid replication origin may be arranged in any order in the expression vector.

The expression vector of the invention may be introduced into a host by any conventional method for transformation of E. coli, e.g., one described in Molecular Cloning, 250-253 (1982), or of Bacillus subtilis, e.g., one described in Molec. Gen. Genet., 168, 111-115 (1979) or Proc. Nat. Acad. Sci. U.S.A., 44, 1072-1078 (1958).

The resulting transformant may be cultured in any conventional medium, e.g. one described in Molecular Cloning, 68-73, (1972), at a temperature in the range of 28 to 42°C in both cases of E. coli and Bacillus subtilis. Preferably, it may be cultured at a temperature in the range of 28 to 30°C where no expression of heat shock proteins may be induced.

The desired protein thus produced may be easily purified from the host in conventional procedures. For example, the host cell may be crushed by lysozyme-surfactant or ultra-sonication, and the insoluble fractions which contain the desired non-A non-B hepatitis-specific antigen may be then collected by centrifugation, solubilized in a surfactant such as 0.01% SDS, and subjected to column chromatography using a monoclonal anti-

body (Japanese Patent Application Laying-open (KOKAI) Nos. 56196/86 and 176856/86.

When an eukaryotic cell such as an animal cell is employed as a host, the expression vector of the invention is preferably as follows :

The promoters used in the vector of the invention for the expression in eukaryotic cells may herein include SV40 early and late promoters ; promoters of apolipoprotein E and A-I genes ; promoter of heat shock protein gene (Proc. Natl. Acad. Sci. U.S.A., 78, 7038-7042 (1981)) ; promoter of metallothionein gene (Proc. Natl. Acad. Sci. U.S.A., 77, 6511-6515 (1980)) ; HSV TK promoter ; adenovirus promoter, such as Ad2 major late promoter (Ad2 MLP) ; LTR (long terminal repeat) of retrovirus ; etc. SV40 promoter and promoter of metallothionein gene are preferred.

The expression vector of the invention may contain a splice sequence comprising 5' splice junction donor site, an intron and 3' splice junction acceptor site. A common base sequence is found at all the splice junction sites (exonintron junction sites) ; so-called GT/AG rule that any intron region always starts from two bases GT at the donor site and terminates at two bases AG of the acceptor site has been established.

The expression vector of the invention may contain one or more splice sequences as mentioned just above. Such splice sequences may be positioned upstream or downstream of the structural gene for a non-A non-B hepatitis-specific antigen.

Illustrative examples of such splice sequences may include those DNA sequences found in exons 2 and 3 of rabbit β-globin gene (Science, 26, 339 (1979)) and mouse methallothionein-I gene containing the promoter, exons 1, 2 and 3 and introns A and B of methallothionein gene (Proc. Natl. Acad. Sci. U.S.A., 77, 6513 (1980)). The 5' and 3' splice sites may be derived from the same or different gene ; for example, a sequence in which 5' splice site contained in adenovirus DNA is linked to 3' splice site derived from the gene of Ig variable region can be employed.

The expression vector of the invention also contains a polyadenylation site downstream from the structural gene of a non-A non-B hepatitis-specific antigen. Illustrative examples of the polyadenylation sites may include those derived from SV40 DNA, β-globin gene or methallothionein gene. A combined site of the polyadenylation sites of β-globin gene and SV40 DNA may be employed in the invention.

The expression vector of the invention may also contain a dominant selective marker permitting the selection of transformants. Selective markers which can be used herein may include DHFR gene imparting MTX (methotrexate) resistance to a host ; tk gene of herpes simplex virus (HSV) which permits selection of tk^- strains transformed therewith in HAT medium ; the gene for aminoglycoside 3'-phosphotransferase from E. coli transposon Tn5, which imparts to a host the resistance against 3'-deoxystreptamine antibiotic G418 ; bovine papilloma virus gene permitting morphological discrimination by piled up growth ; and aprt gene.

Alternatively, animal cells transformed with the expression vector of the invention may be selected by the cotransformation even though no selective marker is present in the vector. For this purpose, an animal cell is cotransformed with both the expression vector and a plasmid or other DNA containing a gene for such a selective marker and selected by a phenotypic trait of the gene.

Advantageously, the expression vectors may also contain a plasmid fragment having an origin of replication derived from a bacterium such as E. coli, since such vectors can be cloned in bacteria. Such plasmids may include pBR322, pBR327, pML, etc.

Illustrative examples of plasmid vectors used as sources of the expression vectors according to the invention may include pKCR (Proc. Natl. Acad. Sci. U.S.A., 78, 1528 (1981)), which contains SV40 early promoter, the splice sequence and polyadenylation site derived from rabbit β-globin gene, the polyadenylation site from SV40 early region, and the origin of replication and ampicillin resistant gene from pBR322 ; pKCR H2 (Nature, 307, 605 (1984)), in which the pBR322 portion of pKCR has been substituted by pBR327 fragment and the EcoRI site present in the exon 3 of rabbit β-globin gene has been converted into HindIII site ; and pBPV MT1 containing BPV gene and methallothionein gene (Proc. Natl. Acad. Sci. U.S.A., 80, 398 (1983)).

Animal cells transformed with the expression vector of the invention may include CHO cells, COS cells, and mouse L cells, C127 cells and FM3A cells.

The introduction of the expression vector of the invention into an animal cell may be carried out by transfection, microinjection, etc. Most often, the transfection may employ $CaPO_4$: Virology, 52, 456-467 (1973).

Animal cells transformed by introducing the expression vector of the invention may be cultured in a suspension or solid medium by conventional methods. The culture medium used is most often MEM, RPMI1640, etc.

Proteins produced in the transformed animal cells can be separated and purified in the almost same manner as in the case of microorganisms aforementioned.

As stated, the invention provides a transformant cell obtained by introducing the expression vector of the invention into a host cell.

Also provided according to the invention is a process for producing a non-A non-B hepatitis-specific antigen

comprising culturing said transformant and collecting the produced and accumulated antigen.

As stated previously, a large amount of an antigenic protein specific to non-A non-B hepatitis is required when such a protein is to be utilized as a direct diagnostic agent. According to the present invention, such an antigenic protein can be produced with a low cost and a large scale without use of infected chimpanzee hepatocytes. Prior to the present invention, it has been difficult obtain such a large amount of a non-A non-B hepatitis-specific antigenic protein from hepatocytes of chimpanzees affected with non-A non-B hepatitis.

Further, the DNA fragment coding for an antigenic protein of non-A non-B hepatitis virus according to the present invention will be useful as a probe for detecting the gene of said antigenic protein by nucleic acid hybridization.

EXAMPLES

The following examples will be given by way of illustration but these examples in no way limit the scope of the invention without departing the concept thereof.

EXAMPLE 1 : Preparation of cDNA Fragment Coding for Antigenic Protein Specific to Non-A Non-B Hepatitis

Poly(A)-containing RNA was prepared from chimpanzee liver according to the guanidine thiocyanate-lithium chloride method : Cathala et al., DNA, 2, 329 (1983).

The infected liver (5 g) was taken out from a chimpanzee affected with non-A non-B hepatitis and immediately frozen by liquid nitrogen. The frozen liver was added into a Waring blender together with liquid nitrogen and ground at 3,000 rpm for 2 minutes. The ground liver specimen was further ground by a Teflon homogenizer at 5 rpm in 100 ml of a solution : 5 M guanidine thiocyanate, 10 mM EDTA, 50 mM Tris-HCl (pH 7), 8% (v/v) β-mercaptoethanol. The thus solubilized material (20 ml) was slowly placed on 5.7 M CsCl solution (10 ml) contained in a centrifuge tube and centrifuged at 27,000 rpm for 20 hours in Hitachi RPS 28-2 rotor. The thus precipitated RNA was collected and dissolved in 10 ml of a solution : 0.1% sodium laurylsulfate, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5). The RNA was extracted with phenol-chloroform and recovered by ethanol precipitation.

The thus obtained RNA (about 3.95 mg) was dissolved in 1 ml of a solution : 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The solution was incubated at 65°C for 5 minutes, and 5 M NaCl (0.1 ml) was added. The resulting mixture was subjected to chromatography on an oligo(dT) cellulose column (column volume of 0.5 ml, P-L Biochemical). The thus adsorbed poly(A)-containing mRNA was eluted with a solution : 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. There was obtained about 100 μg of poly(A)-containing mRNA.

The thus obtained poly(A)$^+$ mRNA (10 μg) was dissolved in 50 μl of RT buffer : 20 mM Tris-HCl (pH 8.8), 0.1 M KCl, 12 mM $MgCl_2$, 2 mM $MnCl_2$. To this solution, there was added 8 μg of random primer $d(N)_6$ (P-L Biochemical). The resulting mixture was heated at 95°C for 3 minutes to denature the materials, which was then cooled gradually to room temperature to anneal the random primer with the mRNA. To the annealed mixture, there were aded 10 mM 4NTP (10 μl) and reverse transcriptase (225 units) from TAKARA SHUZO (Japan), and then water was added so as to make the total volume of the mixture to 100 μl. Reaction was allowed to proceed at 42°C for one hour.

To the reaction mixture (50 μl), there were added 10 mM NAD (2 μl), 10 mM 4dNTP (10 μl), RNase H (5 units), E. coli ligase (1 unit), E. coli DNA polymerase I (6.3 units), and 10x T4 DNA ligase buffer (10 μl ; 0.1 M Tris-HCl, pH 7.5, 0.1 M DTT, 60 mM $MgCl_2$) to make the total volume to 100 μl. The mixture was allowed to react at 37°C for one hour to synthesize a double stranded DNA.

The thus obtained double stranded DNA was extracted with an equal volume of water-saturated phenol. Phenol in the aqueous layer was removed with the aid of ether followed by ethanol precipitation. The precipitate thus obtained was dissolved in 50 μl of water, and 10x T4 DNA polymerase buffer (10 μl ; 0.33 M Tris-acetic acid, pH 7.9, 0.66 M potassium acetate, 0.1 M magnesium acetate, 5 mM DTT), 10 mM 4dNTP (10 μl), and T4 DNA polymerase (6 units) were added to make the total volume to 100 μl. The mixture was reacted at 37°C for one hour. There was obtained a double stranded DNA having blunt ends, which was then extracted with phenol to remove proteins and purified by ethanol precipitation as described above. The thus purified DNA was then air dried.

To the purified DNA, there were added 50 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, 5 mM DTT (20 μl), 100 μM S-adenosyl-L-methionine (2 μl), and 1.8 mg/ml EcoRI methylase (0.2 μl). Reaction was effected at 37°C for 15 minutes, whereby methylating the EcoRI restriction enzyme cleavage site on the DNA fragment. The reaction mixture was then heated at 70°C for 15 minutes to deactivate the enzyme.

To the reaction mixture, there was added 3'-phosphorylated EcoRI linker (GGAATTCC) in an amount of

100 molecules thereof per molecule of the synthetic DNA. There were further added 10x T4 DNA ligase buffer (5 µl ; 0.5 M Tris-HCl, pH 7.5, 60 mM $MgCl_2$, 10 mM DTT), 0.1 M ATP (5 µl), and T4 DNA ligase (5 units) to make the total volume to 50 µl. The resulting reaction mixture was reacted at 4°C for 16 hours followed by heating at 70°C for 10 minutes to deactivate the enzyme. Then, 10x EcoRI buffer (10 µl ; 15 M Tris-HCl, pH 7.5, 0.5 M NaCl, 60 mM $MgCl_2$), and EcoRI (100 units) were added to make the total volume to 100 µl, and the reaction mixture was reacted at 37°C for 2 hours to cut the linker. The reaction mixture was passed through Bio Gel A-50 (0.2 cm × 32 cm, Bio RAD). Elution was effected by a buffer : 10 mM Tris-HCl (pH, 7.5), 6 mM $MgCl_2$. Excess EcoRI linker was removed and, thus, a double stranded cDNA having EcoRI sites at both ends thereof was purified.

To the thus obtained double stranded cDNA fragment having EcoRI sites at both ends, there were added gt 11 DNA (10 µg) cleaved with EcoRI, 10x T4 DNA ligase buffer (10 µl) as described above, 0.1 M ATP (10 µl), and T4 DNA ligase (10 units) to make the total volume to 100 µl. The mixture was reacted at 4°C for 16 hours. Thus, said double stranded cDNA fragment was inserted into λgt 11 DNA.

The λ phage packaging kit (PROMEGA, Biotech) was used to introduce said DNA into λ phage particle. The procedures for packaging were effected according to the instructions of the kit.

The λgt 11 phage having said DNA packaged thereinto was used to transfect E. coli strain Y1090 to form plaques according to the conventional Tomizawa et al. methods described in "Experimental Procedures for Bacteriophages", pp. 99-174, published May 30, 1970 by Iwanami Shoten (Japan). Among about 200,000 plaques, one positive clone was selected by immunological screening as described hereinbelow. A monoclonal antibody used in the immunological screening was prepared by the method described in Japanese Patent Application Laying-open (KOKAI) No. 91328/88.

E. coli Y1090 (R.A. Young et al., Pro. Natl. Acad. Sci. U.S.A., 80, 1194-1198 (1983), which had been transfected with λgt 11, was inoculated in a petri dish together with soft agar held at 42°C. The transfected cell was allowed to stand at 42°C for 5 hours. A nitrocellulose filter (S & S, BA-83, pore size of 0.2 µm) containing 10 mM IPTG was placed on the cell in the dish and incubation was effected at 37°C for 3-4 hours. This nitrocellulose filter was lightly rinsed with TBS buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl), immersed in the TBS buffer (400 ml) containing 3% gelatine and shaked at 40°C for one hour. Thus, the nitrocellulose filter was blocked. Then, a monoclonal antibody (OD_{280} = 4.3) directed to a non-A non-B hepatitis-specific antigen was added to TBS buffer containing 1% gelatine with a dilution of 1/400. This mixture was put into a vinyl bag together with the filter in a proportion of 2 ml of the mixture per filter, and reaction was allowed to proceed at room temperature for 16 hours. The reaction mixture was three times washed with TBS buffer (400 ml) containing 0.05% Tween 20 over 10 minutes. A labelled secondary antibody, anti-mouse IgG-PAP (horseradish peroxidase, Bio Rad) was added to TBS buffer containing 1% gelatine with a dilution of 1/1,000. This mixture and the filter were put into a vinyl bag with a proportion of 2 ml of the mixture per filter. Reaction was allowed to proceed at room temperature for 2 hours. The reaction mixture was three times washed with TBS buffer (400 ml) containing 0.05% Tween 20 over 10 minutes, in the same manner as described above. Color development was effected by dipping the filter and 4-chloro-1-naphthol (12 mg, Bio Rad) into 20 ml of TBS buffer containing hydrogen peroxide. After completion of the color development, the filter was thoroughly washed with water and put into a vinyl bag containing water. The bag was stored in a dark and cold place.

Thus, one positive plaque was obtained. The plaque was three times subjected to single plaque isolation. In each time, immunological screening was effected in the same manner as described above, verifying that the plaque was in fact positive.

The phage was then cultured in a large scale to purify the DNA in the following manner : First, E. coli Y1090 was cultured overnight in 10 ml of NZ medium prepared by adding NZ amine (10 g), NaCl (5 g) and 5 mM $MgCl_2$ to one liter of water followed by adjusting the pH to 7.2. The culture (1 ml) was transfected with the phage, with the m.o.i. (multiplicity of infection) being 0.1. The transfected culture was allowed to stand at 37°C for 10 minutes and then transferred to one liter of NZ medium. Shaking culture was effected at 37°C for 7-8 hours until the cells were lysed. Chloroform (5 ml) was added to the culture and shaking was continued for additional 30 minutes. The culture was subjected to centrifugation at 6,500 rpm for 10 minutes to remove cell debris.

NaCl (29 g) and polyethylene glycol (70 g) were added to and thoroughly dissolved in the obtained supernatant, and the solution was allowed to stand at 4°C overnight. The precipitate was collected by centrifugation at 6,500 rpm for 20 minutes, drained thoroughly, and dissolved in 20 ml of TM buffer : 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$. DNase I and RNase A were added to the solution, both with a concentration of 10 µg/ml, and the reaction was effected at 37°C for one hour. Chloroform (20 ml) was then added to the reaction mixture and stirred ; thus, polyethylene glycol was distributed in the chloroform layer which was then separated from the aqueous layer. This aqueous layer was ultra-centrifuged at 28,000 rpm for 60 minutes. Thus, a pellet of phage particles was obtained.

This pellet was dissolved in TM buffer (1 ml) and subjected to CsCl density gradient centrifugation at 33,000

rpm for 20 hours. The resultant fraction containing the phage particles (ρ = 1.45-1.50) was dialyzed overnight against TM buffer. Proteinase K was added to the dialyzate in an amount of 100 µg/ml and reaction was effected at 37°C for one hour. Thereafter, an equal volume of water-saturated phenol was added and phenol-extraction was gently effected. After centrifugation at 6,500 rpm for 10 minutes, the aqueous layer was removed, put into a dialysis tube, and dialyzed overnight against water at 4°C. Thus, about 5 mg of DNA was obtained.

Cleavage reaction of this DNA (100 µg) with EcoRI (100 units) in the aforementioned buffer (100 µl) at 37°C revealed that two cDNA segments of 390 bp and 345 bp were inserted into the phage DNA.

These two EcoRI fragments were re-cloned into EcoRI site of a cloning vector pUC 119. Base sequences of these DNA fragments were determined by the dideoxy method using commercially available primers CAGGAAACAGCTATGAC and AGTCACGACGTTGTA, respectively. The base sequence of the linking portion between these two DNA fragments was similarly determined by cutting this cDNA fragment at BamHI and EcoRV sites present therein with corresponding specific restriction enzymes, inserting the resulting BamHI-EcoRV DNA fragment between BamHI and SmaI sites of the plasmid pUC 119, and sequencing the fragment by the dideoxy method.

The base sequence of said cDNA fragment is shown in Fig. 3. This was a partial cDNA fragment of a gene coding for an antigenic protein specific to non-A non-B hepatitis.

EXAMPLE 2 : Preparation of cDNA Containing the Full Length Gene Sequence

Messenger RNA was prepared as described in Example 1 and cDNA was synthesized using Okayama vector according to the conventional method described in Molecular Cloning, p. 211 et seq. The procedures used to synthesize cDNA were as follows :

To 300 µl of a solution (10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl), there were added 400 µg of pCDV 1 (Okayama and Berg, Mol. Cell. Biol., 3, 280 (1983)) and 500 units of KpnI (TAKARA SHUZO, Japan), all restriction enzymes used hereinafter having been manufactured by TAKARA SHUZO (Japan) unless otherwise noted. Reaction was effected at 37°C for 6 hours to cut the plasmid at KpnI site therein. After phenol-chloroform extraction, ethanol precipitation was effected to recover DNA.

The DNA (about 200 µg) cleaved with KpnI was added to 200 µl of a solution which was obtained by adding dTTP in a concentration of 0.25 mM to a buffer (TdT buffer) : 40 mM sodium cacodylate, 30 mM Tris-HCl (pH 6.8), 1 mM $CaCl_2$, 0.1 mM dithiothreitol (DTT). Further, 81 units of terminal deoxynucleotidyl transferase (TdT, manufactured by P-L Biochemicals) was also added. Reaction was effected at 37°C for 11 minutes. Thus, a poly(dT) chain (about 67 deoxythymidylic acid residues) was added to the 3′ end at the KpnI-cleaved site of pCDV 1. After phenol-chloroform extraction and ethanol-precipitation, about 100 µg of pCDV 1 DNA to which poly(dT) chain had been added was recovered from the reaction mixture.

The thus obtained DNA was added to 150 µl of a buffer (10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl), and HpaI (360 units) was also added, followed by reaction at 37°C for 2 hours. The reaction mixture was subjected to electrophoresis on agarose gel to separate and recover about 3.1 Kbp DNA fragment. Thus, there was obtained about 60 µg of poly(dT)-containing pCDV 1.

The thus obtained DNA was dissolved in 500 µl of a solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), incubated at 65°C for 5 minutes, and cooled on ice. After adding 5 M NaCl (50 µl), the mixture was subjected to chromatography on oligo(dA) cellulose column (Colaborative Research). DNA having a poly(dT) chain of sufficient length was adsorbed on the column and eluted with a solution : 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. Thus, there was obtained 27 µg of pCDV 1 to which poly(dT) chain had been added, abbreviated hereinafter as vector primer.

A linker DNA was prepared in the following manner : To 200 µl of a solution (10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 50 mM NaCl), there were added about 14 µg of pL 1 (Okayama and Berg, Mol. Cell. Biol., 3, 280 (1983)) and 50 units of PstI. Reaction was effected at 37°C for 4 hours to cut the pL 1 DNA at PstI site. Phenol-chloroform extraction and ethanol precipitation of the reaction product gave about 13 µg of pL 1 DNA cleaved at PstI site.

The thus obtained DNA (about 13 µg) was added to 50 µl of the TdT buffer containing dGTP at a final concentration of 0.25 mM, and 54 units of TdT (P-L Biochemicals) was also added. The mixture was incubated at 37°C for 13 minutes to add a (dG) chain (about 14 deoxyguanylic acid residues) to the 3′ end at the PstI-cleaved site of pL 1. After phenol-chloroform extraction, DNA was recovered by ethanol precipitation.

The thus obtained DNA was added to 100 µl of a buffer (10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 60 mM NaCl), and 80 units of HindIII was also added. The mixture was incubated at 37°C for 3 hours to cut the pL 1 DNA at HindIII site. The reaction product was fractionated by agarose gel electrophoresis. About 0.5 Kb DNA fragment was recovered by the DEAE paper method : Dretzen et al., Anal. Biochem., 112, 295 (1981). Thus, there was obtained an oligo(dG) chain-containing linker DNA, hereinafter abbreviated simply as linker DNA.

The aforementioned poly(A)$^+$ RNA (about 2 μg) prepared in the same manner as in Example 1 and the vector primer (about 1.4 μg) were dissolved in 22.3 μl of a solution : 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 0.3 mM DTT, 2 mM dNTP (dATP, dTTP, dGTP and dCTP) and 10 units of ribonuclease inhibitor (P-L Biochemicals). To the solution, there was added 10 units of reverse transcriptase manufactured by SEIKAGAKU KOGYO (Japan). Incubation was effected at 37°C for 40 minutes to synthesize a DNA complementary to the mRNA. After phenol-chloroform extraction and ethanol precipitation, the vector primer DNA to which a double stranded RNA-DNA had been added was recovered.

The thus obtained vector primer DNA containing RNA-DNA double stranded chain was dissolved in 20 μl of TdT buffer containing 60 μM dCTP and 0.2 μg poly(A). After adding 14 units of TdT (P-L Biochemical), the mixture was incubated at 37°C for 8 hours to add a (dC) chain of 12 deoxycytidylic acid residues to the 3' end of the cDNA. The reaction product was extracted with phenol-chloroform and precipitated with ethanol to recover a cDNA-vector primer DNA to which a (dC) chain had been added.

The thus obtained (dC) chain-containing cDNA-vector primer DNA was dissolved in 400 μl of a solution (10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 60 mM NaCl), and 20 units of HindIII was also added. The mixture was incubated at 37°C for 2 hours to cut the DNA at HindIII site. The reaction product was extracted with phenol-chloroform and precipitated with ethanol. Thus, there was obtained 0.5 pmole of a (dC) chain-containing cDNA-vector primer DNA.

The thus obtained (dC) chain-containig cDNA-vector primer DNA (0.08 pmole) and the aforementioned linker DNA (0.16 pmole) were dissolved in 40 μl of a solution : 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA. The resulting solution was incubated at 65°C for 10 minutes, at 42°C for 25 minutes, and then at 0°C for 30 minutes. The reaction mixture was adjusted to 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1 M KCl and 0.1 mM β-NAD in a total volume of 400 μl.

To the reaction mixture, there was added 10 units of E. coli DNA ligase (New England Biolabs), followed by incubation overnight at 11°C. After adjusting the concentrations of dNTP and β-NAD in the reaction mixture to 40 μM and 0.15 mM, respectively, by supplementing necessary reagents, 5 units of E. coli DNA ligase, 7 units of E. coli DNA polymerase I (P-L Biochemicals) and 2 units of E. coli ribonuclease H (P-L Biochemicals) were added to the reaction mixture. The mixture was incubated at 12°C for one hour and then at 25°C for one hour.

In the course of the above reactions, a recombinant DNA containing the cDNA was cyclized and the RNA portion of the RNA-DNA double stranded chain was substituted by DNA. Thus, a desired recombinant plasmid containing a complete double-stranded DNA was produced.

The recombinant plasmid was used to transform competent cells of E. coli strain MC1064 prepared by conventional methods. Approximately 50,000 transformants were fixed on a nitrocellulose filter. These colonies were screened according to the colony hybridization method described in Molecular Cloning, Cold Spring Harbor Laboratory, p. 329 et seq. (1982) using the cDNA fragment obtained in Example 1 as a ^{32}P-labelled probe. Thus, three clones showed strong hybridization at 42°C.

These positive clones were analyzed in detail by Southern method : J. Mol. Biol., 98, 503 (1975). There was obtained the desired full length cDNA of a gene coding for an antigenic protein specific to non-A non-B hepatitis. The base sequence of the cDNA is shown in Fig. 4.

The expression vector containing the full length cDNA was designated as pCDVCL-I.

EXAMPLE 3 : Preparation of Expression Vector and Transformant and Expression of Specific Antigen

A. Preparation of Expression Vector and Transformant

I) Modification of N-terminus (Fig. 5) :

i) In 100 μl of a buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$), pCDVCL-I (5 μg) was digested with PvuI (10 units) at 37°C for 2 hours. The reaction mixture was heated at 75°C for 15 minutes to deactivate the enzyme, dialyzed against water, and dried. The cleaved plasmid DNA was treated with T4 DNA polymerase (4 units) in 40 μl of a system : 33 mM Tris-acetic acid (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate and 0.5 mM dithiothreitol, to which 2 mM 4-deoxytriphosphate had been added ; thus, the 3' protruding end of the plasmid DNA was filled in to produce a blunt end. The thus treated mixture was heated at 70°C for 10 minutes to deactivate the enzyme, dialyzed against water, and dried. The thus obtained plasmid DNA was then stored in the form of an aqueous solution (50 μl). This plasmid DNA fragment is hereinafter designated as Fragment I.

ii) On the other hand, pCDVCL-I (20 μg) was digested with NcoI and HindIII (each 20 units) at 37°C for 2 hours in 100 μl of a buffer : 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM $MgCl_2$. The plasmid DNA was

subjected to 5% acrylamide gel electrophoresis at 10 V/cm for 1.5 hours in a buffer : 89 mM Tris, 89 mM boric acid, 2 mM EDTA. The gel was stained with 0.05% aqueous ethidium bromide solution and two gel slices corresponding to DNA fragments of larger molecular weights were excised from the gel under ultraviolet radiation at 340 nm. The gel slices were crushed by means of a glass rod, suspended into 4 ml of a buffer for DNA extraction (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% sodium laurylsulfate), and allowed to stand overnight at 37°C to extract DNA from the gel. The materials were subjected to centrifugation at 10,000 rpm for 15 minutes to eliminate larger gel pieces, and passed through a glass filter to remove smaller gel pieces. The DNA was purified by effecting ethanol precipitation three times and stored in the form of an aqueous solution (200 µl). This plasmid DNA fragment is hereinafter designated as Fragment II.

iii) A primer of the DNA portion to be modified as shown below (51 bases) was synthesized by a DNA synthesizer, NIKKAKI (Japan), Applied Biosystem MODEL 380A. The synthesized DNA was overnight reacted with concentrated aqueous ammonia at 55°C to deprotect and purified by reversed HPLC before use.

```
                                    HindIII
Primer                  ACAACAGATCTAAGCTTATGGCAGTTACAACAAGATTAA
                                  x  x x          x      xx x  x
(Original sequence) (-----------A--A-G----------G------TC-C--G-

                        CATGGTTGCATG  wherein x represents a
                                      base substitution.
                        ------------)
```

The synthetic primer (150 pmole) was treated with T4 polynucleotide kinase (20 units) in 10 µl of a kinase buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol) to phosphorylate the 5' end thereof.

iv) Fragment I (0.05 pmole), Fragment II (0.05 pmole) and 5'-phosphorylated primer (45 pmole) were added to 12 µl of 5x polymerase-ligase buffer (0.5 M NaCl, 32.5 mM Tris-HCl, pH 7.5, 40 mM $MgCl_2$, 5 mM β-mercaptoethanol) to make the total volume of the mixture 34.8 µl. The mixture was boiled at 100°C for 3 minutes, immediately after which it was placed in a thermostat at 30°C and allowed to stand for 30 minutes. The mixture was allowed to stand at 4°C for 30 minutes and then on ice for 10 minutes to form a heteroduplex.

To an aqueous solution (11.6 µl) containing the heteroduplex, there were added 2.5 mM 4-deoxynucleotide triphosphate (2 µl), 10 mM ATP (2 µl), Klenow enzyme (2 units) and T4 DNA ligase (0.5 units) to form a mixture of 20 µl in total volume. The mixture was reacted overnight at 16°C to cyclize the DNA.

An aqueous solution (2 µl) containing the circular DNA was used to transform E. coli HB101 strain according to conventional methods. Plasmids were separated from the transformant and purified in conventional manners. The plasmid was cleaved with restriction enzyme HindIII and subjected to 5% acrylamide gel electrophoresis. Thus, two separate fragments were collected as desired modified, variant plasmids. Since resulting variant plasmids might often be admixed with original wild-type plasmids, the thus obtained variant plasmids were again employed to transform E. coli HB101 so as to purify the plasmid.

Thus, a purified plasmid pCV44H was obtained (Fig. 5).

II) Modification of C-terminus (Fig. 6) :

i) Plasmid pCDVCL-I (5 µg) was treated in the same manner as in I) i) described above to produce Fragment I.

ii) Plasmid pCDVCL-I (20 µg) was treated in the same manner as in I) ii) described above except that NcoI and NsiI (each 5 units) were employed. Thus, Fragment II was produced.

iii) In the same manner as in I) iii) described above, the following primer (46 bases) was synthesized and the 5' end thereof was phosphorylated.

```
                                          BglII   SalI
Primer                    GCACAAGGAAAAAAATGAGATCTGTCGACGGTTCACGTA
                                          xxxxxxxxxxxxx
(Original sequence) (----------------AGATATGTGAA*A----------

                          AATTTCC  wherein x represents a base
                                   substitution and * represents
                          -------) an addition.
```

iv) The Fragment I and II and the 5'-phosphorylated primer obtained above in II) i) to iii) were treated in the same manner as in I) iv) described above. Thus, plasmid pCV44B was obtained (Fig. 6).

III) Introduction of cDNA coding for specific antigen into expression vector (Fig. 7) :

i) In 100 µl of a buffer H (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$), 10 µg (about 3 pmole) of pCV44H was cut with HindIII (20 units) and SacI (20 units) at 37°C for 2 hours. The reaction mixture was subjected to 5% acrylamide gel electrophoresis. Thus, a 467 bp DNA fragment coding for the N-terminus of the specific antigen was separated and purified. This fragment is hereinafter designated as Fragment N.

ii) In 100 µl of the buffer H, 10 µg (about 3 pmole) of pCV44B was cleaved with BglII (20 units) and SacI (20 units) at 37°C for 2 hours. The reaction mixture was subjected to 5% acrylamide gel electrophoresis to isolate and purify a 836 bp DNA fragment coding for the C-terminus of the specific antigen. The thus obtained fragment is hereinafter designated as Fragment C.

iii) In 20 µl of buffer H, 2 µg (about 1 pmole) of an expression vector pUSΔH was cut with HindIII (2 units) and BglII (2 units) at 37°C for 2 hours. The reaction mixture was extracted with an equal volume of water-saturated phenol to remove proteins. After extracting the phenol with ether, the reaction mixture was dialyzed against water to desalt, and concentrated by a vacuum pump. Thus, there was obtained 10 µl of an aqueous solution containing an expression vector fragment HB.

iv) Fragment N (0.5 pmole), Fragment C (0.5 pmole) and the expression vector fragment HB (0.1 pmole) were mixed and reacted with T4 DNA ligase (1 unit) at 4°C for 16 hours in 10 µl of a buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 6 mM $MgCl_2$, 1 mM ATP). The reaction mixture (3 µl) was used to transform commercially available E. coli JM109 competent cell according to conventional methods. The resulting transformants were selected in L broth plate (bactopeptone 10 g, yeast exstract 5 g, NaCl 10 g, agar 15 g per liter) containing 20 µg/ml ampicillin. Thus, there was obtained an expression vector pCZ44 containing the specific antigen gene inserted thereinto (Fig. 7).

B. Expression of Specific Antigen

E. coli strain JM109 possessing pCZ44 was cultured overnight at 30°C in L broth. The culture was inoculated in a fresh L broth with a dilution of 1/50 and cultured with shaking at 30°C for 2 hours. After IPTG (isopropylthio-β-D-galactopyranoside) was added to the medium in a concentration of 2 mM, shaking culture was continued at 30°C for further 3 hours. The cells were collected by centrifugation at 6,500 rpm for 10 minutes and suspended in a buffer (0.9% NaCl, 10 mM Tris-HCl, pH 7.5) to store.

C. Verification of Expression of Specific Antigen

The thus obtained cell culture (0.3 ml) was subjected to 10% SDS polyacrylamide gel electrophoresis at 120 V for one hour in a buffer (Tris 3g/l, glycine 14.4 g/l, 0.1% SDS). The gel was removed, placed on a nitrocellulose filter, interposed between filter papers and electrophoresed at 5 V/cm, 4°C in a buffer (Tris 3g/l, glycine 14.4 g/l) to transferred proteins in the gel onto the nitrocellulose filter. The nitrocellulose filter was rinsed with TBS buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl), immersed into 400 ml of TBS buffer containing 3% gelatine and shaked at 40°C for one hour to block the nitrocellulose filter.

To TBS buffer containing 1% gelatine, there was added a monoclonal antibody directed to a non-A non-B hepatitis-specific antigen (OD_{280} = 4.3) with a dilution of 1/400. The resulting mixture and the nitrocellulose filter were put into a vinyl bag so that the mixture was present in an amount of 2 ml per filter. Reaction was effected at room temperature for 16 hours. The reaction mixture was washed three times with 400 ml of TBS buffer con-

taining 0.05% Tween 20 for 10 minutes.

To TBS buffer containing 1% gelatine, there was added a labelled secondary antibody, anti-mouse IgG-PAP (horseradish peroxidase, Bio Rad), with a dilution of 1/1000. The resulting mixture and the nitrocellulose filter were put into a vinyl bag so that the mixture was present in an amount of 2 ml per filter. Reaction was effected at room temperature for 2 hours. The reaction mixture was washed three times with 400 ml of TBS buffer containing 0.05% Tween 20 for 10 minutes.

Color formation was effected by immersing the filter into 20 ml of TBS buffer containing 12 mg of 4-chloro-1-naphthol (Bio Rad) and hydrogen peroxide. After completion of color formation, the filter was thoroughly washed with water, put into a vinyl bag containing water, and stored in a dark and cold place.

Such a test effected showed that a protein reacting with the monoclonal antibody was found at the same position (44 Kd) as found in the case of the specific antigen derived from infected chimpanzee liver. This verifies that such a specific antigen can be in fact expressed in E coli. The invention thus also relates to a process for the in vitro diagnosis of NON-A NON-B hepatitis, which comprises contacting a liver sample and/or a serum sample taken from a patient possibly infected with a NON-A NON-B hepatitis with the protein whose formula appears in claim 3 hereafter or a part thereof for a time and under conditions sufficient to allow for the production of a complex between said protein or part thereof with the antibodies contained in the patient sample and detecting the presence of the immunologic complex, particularly when the patient is suffering from NON-A NON-B hepatitis.

Any part of said protein,or any recombinant, produced by genetic engineering and including the aminoacid sequence of said protein or part of said protein can be substituted for above-said protein, it being understood that the said recombinant protein or part of said protein are specifically recognized by the same antibodies as those which recognize said protein.

In other words the invention relates to all recombinant proteins or protein fragments which bind to antibodies contained in a liver extract or serum sample, or both, and originating from a patient suffering from NON-A NON-B hepatitis.

The invention also relates to a process for detecting in vitro an infection by a NON-A NON-B hepatitis virus, which process comprises contacting the DNA of claim 4, or a fragment thereof, under suitable hybridization conditions, with a sample of liver extract and/or serum sample originating from the patient to be diagnosed and in which the nucleic acid components had previously been made accessible to hybridization, to form a hybridization product between said DNA of claim 4 (probe) and the viral DNA of a NON-A NON-B hepatitis B virus, and detecting said hybridization product, particularly in the case where the patient is indeed infected with a NON-A NON-B virus.

Claims

1. A DNA fragment which contains a base sequence coding for an antigenic protein specifically occurring in a host affected with non-A non-B hepatitis, said protein comprising the whole or a part of the amino acid sequence represented by the formula :

Met Ala Val Thr Thr Arg Leu Thr Trp Leu His Glu Lys Ile Leu
10

Gln Asn His Phe Gly Gly Lys Arg Leu Ser Leu Leu Tyr Lys Gly
20 30

Ser Val His Gly Phe His Asn Gly Val Leu Leu Asp Arg Cys Cys
40

Asn Gln Gly Pro Thr Leu Thr Val Ile Tyr Ser Glu Asp His Ile
50 60

Ile Gly Ala Tyr Ala Glu Glu Gly Tyr Gln Glu Arg Lys Tyr Ala
70

Ser Ile Ile Leu Phe Ala Leu Gln Glu Thr Lys Ile Ser Glu Trp
80 90

Lys Leu Gly Leu Tyr Thr Pro Glu Thr Leu Phe Cys Cys Asp Val
100

Ala Lys Tyr Asn Ser Pro Thr Asn Phe Gln Ile Asp Gly Arg Asn
110 120

Arg Lys Val Ile Met Asp Leu Lys Thr Met Glu Asn Leu Gly Leu
130

Ala Gln Asn Cys Thr Ile Ser Ile Gln Asp Tyr Glu Val Phe Arg
140 150

Cys Glu Asp Ser Leu Asp Glu Arg Lys Ile Lys Gly Val Ile Glu
160

Leu Arg Lys Ser Leu Leu Ser Ala Leu Arg Thr Tyr Glu Pro Tyr
170 180

Gly Ser Leu Val Gln Gln Ile Arg Ile Leu Leu Leu Gly Pro Ile
190

```
                                200                                 210
Gly Ala Gly Lys Ser Ser Phe Phe Asn Ser Val Arg Ser Val Phe

                                                220
Gln Gly His Val Thr His Gln Ala Leu Val Gly Thr Asn Thr Thr

                                230                                 240
Gly Ile Ser Glu Lys Tyr Arg Thr Tyr Ser Ile Arg Asp Gly Lys

                                                250
Asp Gly Lys Tyr Leu Pro Phe Ile Leu Cys Asp Ser Leu Gly Leu

                                260                                 270
Ser Glu Lys Glu Gly Gly Leu Cys Met Asp Asp Ile Ser Tyr Ile

                                                280
Leu Asn Gly Asn Ile Arg Asp Arg Tyr Gln Phe Asn Pro Met Glu

                                290                                 300
Ser Ile Lys Leu Asn His His Asp Tyr Ile Asp Ser Pro Ser Leu

                                                310
Lys Asp Arg Ile His Cys Val Ala Phe Val Phe Asp Ala Ser Ser

                                320                                 330
Ile Glu Tyr Phe Ser Ser Gln Met Ile Val Lys Ile Lys Arg Ile

                                                340
Arg Arg Glu Leu Val Asn Ala Gly Val Val His Val Ala Leu Leu

                                350                                 360
Thr His Val Asp Ser Met Asp Leu Ile Thr Lys Gly Asp Leu Ile

                                                370
Glu Ile Glu Arg Cys Val Pro Val Arg Ser Lys Leu Glu Glu Val

                                380                                 390
Gln Arg Lys Leu Gly Phe Ala Leu Ser Asp Ile Ser Val Val Ser

                                                400
Asn Tyr Ser Ser Glu Trp Glu Leu Asp Pro Val Lys Asp Val Leu

                                410                                 420
Ile Leu Ser Ala Leu Arg Arg Met Leu Trp Ala Ala Asp Asp Phe

                                                430
Leu Glu Asp Leu Pro Phe Glu Gln Ile Gly Asn Leu Arg Glu Glu

                                440
Ile Ile Asn Cys Ala Gln Gly Lys Lys ***.
```

2. The DNA fragment in accordance with claim 1, in which the base sequence comprises the whole or a part of the base sequence represented by the formula :

```
                    10                  20                  30                  40
5'  ATG GCA GTG ACA ACT CGT TGG ACA TGG TTG CAT GAA AAG ATC
3'  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                50                  60                  70                  80
CTG CAA AAT CAT TTT GGA GGG AAG CGG CTT AGC CTT CTC TAT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

      90                  100                 110                 120
AAG GGT AGT GTC CAT GGA TTC CAT AAT GGA GTT TTG CTT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

  130                 140                 150                 160
AGA TGT TGT AAT CAA GGG CCT ACT CTA ACA GTG ATT TAT AGT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

170                 180                 190                 200                 210
GAA GAT CAT ATT ATT GGA GCA TAT GCA GAA GAG GGT TAC CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

          220                 230                 240                 250
GAA AGA AAG TAT GCT TCC ATC ATC CTT TTT GCA CTT CAA GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

        260                 270                 280                 290
ACT AAA ATT TCA GAA TGG AAA CTA GGA CTA TAT ACA CCA GAA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

    300                 310                 320                 330
ACA CTG TTT TGT TGT GAC GTT GCA AAA TAT AAC TCC CCA ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

```
   340             350             360             370
AAT TTC CAG ATA GAT GGA AGA AAT AGA AAA GTG ATT ATG GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

380             390             400             410             420
TTA AAG ACA ATG GAA AAT CTT GGA CTT GCT CAA AAT TGT ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

        430             440             450             460
ATC TCT ATT CAG GAT TAT GAA GTT TTT CGA TGC GAA GAT TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

    470             480             490             500
CTG GAC GAA AGA AAG ATA AAA GGG GTC ATT GAG CTC AGG AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

510             520             530             540
AGC TTA CTG TCT GCC TTG AGA ACT TAT GAA CCA TAT GGA TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

  550             560             570             580
CTG GTT CAA CAA ATA CGA ATT CTG CTG CTG GGT CCA ATT GGA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

590             600             610             620             630
GCT GGG AAG TCT AGC TTT TTC AAC TCA GTG AGG TCT GTT TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

        640             650             660             670
CAA GGG CAT GTA ACG CAT CAG GCT TTG GTG GGC ACT AAT ACA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

    680             690             700             710
ACT GGG ATA TCT GAG AAG TAT AGG ACA TAC TCT ATT AGA GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

720             730             740             750
GGG AAA GAT GGC AAA TAC CTG CCA TTT ATT CTG TGT GAC TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

  760             770             780             790
CTG GGG CTG AGT GAG AAA GAA GGC GGC CTG TGC ATG GAT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

800             810             820             830             840
ATA TCC TAC ATC TTG AAC GGT AAC ATT CGT GAT AGA TAC CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

        850             860             870             880
TTT AAT CCC ATG GAA TCA ATC AAA TTA AAT CAT CAT GAC TAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

```
        890                 900                 910                 920
ATT GAT TCC CCA TCG CTG AAG GAC AGA ATT CAT TGT GTG GCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

    930                 940                 950                 960
TTT GTA TTT GAT GCC AGC TCT ATT GAA TAC TTC TCC TCT CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

  970                 980                 990                1000
ATG ATA GTA AAG ATC AAA AGA ATT CGA AGG GAG TTG GTA AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

1010                1020                1030                1040                1050
  GCT GGT GTG GTA CAT GTG GCT TTG CTC ACT CAT GTG GAT AGC
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

          1060                1070                1080                1090
  ATG GAT CTG ATT ACA AAA GGT GAC CTT ATA GAA ATA GAG AGA
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

       1100                1110                1120                1130
  TGT GTG CCT GTG AGG TCC AAG CTA GAG GAA GTC CAA AGA AAA
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

    1140                1150                1160                1170
  CTT GGA TTT GCT CTT TCT GAC ATC TCG GTG GTT AGC AAT TAT
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

  1180                1190                1200                1210
  TCC TCT GAG TGG GAG CTG GAC CCT GTA AAG GAT GTT CTA ATT
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

1220                1230                1240                1250                1260
  CTT TCT GCT CTG AGA CGA ATG CTA TGG GCT GCA GAT GAC TTC
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

          1270                1280                1290                1300
  TTA GAG GAT TTG CCT TTT GAG CAA ATA GGG AAT CTA AGG GAG
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

       1310                1320                1330
  GAA ATT ATC AAC TGT GCA CAA GGA AAA AAA 3'
  --- --- --- --- --- --- --- --- --- --- 5'
```

wherein the sign "–" represents a base complementary to the base shown just above each sign.

3. An expression vector in which a DNA fragment containing a base sequence according to claim 1 or 2 and coding for an antigen specifically occurring in a host affected with non-A non-B hepatitis is introduced into a cloning site present downstream from a promoter of said vector.

4. The expression vector in accordance with claim 3, in which the promoter is controllable by a regulatory factor.

5. The expression vector in accordance with claim 3, in which the promoter operates in a microorganism.

6. The expression vector in accordance with claim 3, in which the promoter operates in an eukaryote.

7. A transformant obtained by transforming a host with an expression vector in which a DNA fragment containing a base sequence according to claim 1 or 2 and coding for an antigen specifically occurring in a host affected with non-A non-B hepatitis is introduced into a cloning site present downstream from a promoter of said vector.

8. The transformant in accordance to claim 7, in which the host is Escherichia coli or Bacillus subtilis.

9. A process for producing an antigen occuring specifically in a host affected with non-A non-B hepatitis, comprising introducing a DNA fragment containing a base sequence according to claim 1 or 2 and coding for said specifically occuring antigen into a cloning site present downstream from a promoter of a vector for expression, introducing the expression vector containg said DNA fragment into a host, culturing said transformed, and collecting the produced and accumulated antigen.

10. Process for the in vitro diagnosis of non-A non-B hepatitis which comprises contacting a liver sample and/or a serum sample taken from a patient suspected of being infected with a non-A non-B hepatitis, with the protein whose sequence appears in claim 1, or a part thereof, for a time and under conditions sufficient to allow for the production of a complex between said protein or a part thereof with the antibodies contained in the patient sample and detecting the presence of the immunological complex, particularly when the patient is suffering from non-A non-B hepatitis.

Revendications

1. Fragment d'ADN qui contient une séquence de bases codant pour une protéine antigénique rencontrée spécifiquement chez un hôte atteint de l'hépatite non-A non-B, ladite protéine comprenant la totalité ou une partie de la séquence d'acides aminés représentée par la formule :

```
                                                                  10
Met Ala Val Thr Thr Arg Leu Thr Trp Leu His Glu Lys Ile Leu

                  20                                                  30
Gln Asn His Phe Gly Gly Lys Arg Leu Ser Leu Leu Tyr Lys Gly

                                                                  40
Ser Val His Gly Phe His Asn Gly Val Leu Leu Asp Arg Cys Cys

                  50                                                  60
Asn Gln Gly Pro Thr Leu Thr Val Ile Tyr Ser Glu Asp His Ile

                                                                  70
Ile Gly Ala Tyr Ala Glu Glu Gly Tyr Gln Glu Arg Lys Tyr Ala

                  80                                                  90
Ser Ile Ile Leu Phe Ala Leu Gln Glu Thr Lys Ile Ser Glu Trp

                                                                 100
Lys Leu Gly Leu Tyr Thr Pro Glu Thr Leu Phe Cys Cys Asp Val

                 110                                                 120
Ala Lys Tyr Asn Ser Pro Thr Asn Phe Gln Ile Asp Gly Arg Asn

                                                                 130
Arg Lys Val Ile Met Asp Leu Lys Thr Met Glu Asn Leu Gly Leu

                 140                                                 150
Ala Gln Asn Cys Thr Ile Ser Ile Gln Asp Tyr Glu Val Phe Arg

                                                                 160
Cys Glu Asp Ser Leu Asp Glu Arg Lys Ile Lys Gly Val Ile Glu

                 170                                                 180
Leu Arg Lys Ser Leu Leu Ser Ala Leu Arg Thr Tyr Glu Pro Tyr

                                                                 190
Gly Ser Leu Val Gln Gln Ile Arg Ile Leu Leu Leu Gly Pro Ile
```

```
                                  200                                 210
Gly Ala Gly Lys Ser Ser Phe Phe Asn Ser Val Arg Ser Val Phe

                                                  220
Gln Gly His Val Thr His Gln Ala Leu Val Gly Thr Asn Thr Thr

                                  230                                 240
Gly Ile Ser Glu Lys Tyr Arg Thr Tyr Ser Ile Arg Asp Gly Lys

                                                  250
Asp Gly Lys Tyr Leu Pro Phe Ile Leu Cys Asp Ser Leu Gly Leu

                                  260                                 270
Ser Glu Lys Glu Gly Gly Leu Cys Met Asp Asp Ile Ser Tyr Ile

                                                  280
Leu Asn Gly Asn Ile Arg Asp Arg Tyr Gln Phe Asn Pro Met Glu

                                  290                                 300
Ser Ile Lys Leu Asn His His Asp Tyr Ile Asp Ser Pro Ser Leu

                                                  310
Lys Asp Arg Ile His Cys Val Ala Phe Val Phe Asp Ala Ser Ser

                                  320                                 330
Ile Glu Tyr Phe Ser Ser Gln Met Ile Val Lys Ile Lys Arg Ile

                                                  340
Arg Arg Glu Leu Val Asn Ala Gly Val Val His Val Ala Leu Leu

                                  350                                 360
Thr His Val Asp Ser Met Asp Leu Ile Thr Lys Gly Asp Leu Ile

                                                  370
Glu Ile Glu Arg Cys Val Pro Val Arg Ser Lys Leu Glu Glu Val

                                  380                                 390
Gln Arg Lys Leu Gly Phe Ala Leu Ser Asp Ile Ser Val Val Ser

                                                  400
Asn Tyr Ser Ser Glu Trp Glu Leu Asp Pro Val Lys Asp Val Leu

                                  410                                 420
Ile Leu Ser Ala Leu Arg Arg Met Leu Trp Ala Ala Asp Asp Phe

                                                  430
Leu Glu Asp Leu Pro Phe Glu Gln Ile Gly Asn Leu Arg Glu Glu

                                  440
Ile Ile Asn Cys Ala Gln Gly Lys Lys ***.
```

2. Fragment d'ADN selon la revendication 1, dans lequel la séquence de bases comprend la totalité ou une partie de la séquence de bases représentée par la formule :

```
                    10                  20                  30                  40
5'  ATG GCA GTG ACA ACT CGT TTG ACA TGG TTG CAT GAA AAG ATC
3'  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                50                  60                  70                  80
    CTG CAA AAT CAT TTT GGA GGG AAG CGG CTT AGC CTT CTC TAT
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

          90                  100                 110                 120
    AAG GGT AGT GTC CAT GGA TTC CAT AAT GGA GTT TTG CTT GAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

              130                 140                 150                 160
    AGA TGT TGT AAT CAA GGG CCT ACT CTA ACA GTG ATT TAT AGT
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

    170                 180                 190                 200                 210
    GAA GAT CAT ATT ATT GGA GCA TAT GCA GAA GAG GGT TAC CAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                    220                 230                 240                 250
    GAA AGA AAG TAT GCT TCC ATC ATC CTT TTT GCA CTT CAA GAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

              260                 270                 280                 290
    ACT AAA ATT TCA GAA TGG AAA CTA GGA CTA TAT ACA CCA GAA
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

          300                 310                 320                 330
    ACA CTG TTT TGT TGT GAC GTT GCA AAA TAT AAC TCC CCA ACT
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

```
   340          350          360          370
AAT TTC CAG ATA GAT GGA AGA AAT AGA AAA GTG ATT ATG GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

380          390          400          410          420
TTA AAG ACA ATG GAA AAT CTT GGA CTT GCT CAA AAT TGT ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

             430          440          450          460
ATC TCT ATT CAG GAT TAT GAA GTT TTT CGA TGC GAA GAT TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

        470          480          490          500
CTG GAC GAA AGA AAG ATA AAA GGG GTC ATT GAG CTC AGG AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

     510          520          530          540
AGC TTA CTG TCT GCC TTG AGA ACT TAT GAA CCA TAT GGA TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

   550          560          570          580
CTG GTT CAA CAA ATA CGA ATT CTG CTG CTG GGT CCA ATT GGA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

590          600          610          620          630
GCT GGG AAG TCT AGC TTT TTC AAC TCA GTG AGG TCT GTT TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

             640          650          660          670
CAA GGG CAT GTA ACG CAT CAG GCT TTG GTG GGC ACT AAT ACA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

        680          690          700          710
ACT GGG ATA TCT GAG AAG TAT AGG ACA TAC TCT ATT AGA GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

     720          730          740          750
GGG AAA GAT GGC AAA TAC CTG CCA TTT ATT CTG TGT GAC TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

   760          770          780          790
CTG GGG CTG AGT GAG AAA GAA GGC GGC CTG TGC ATG GAT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

800          810          820          830          840
ATA TCC TAC ATC TTG AAC GGT AAC ATT CGT GAT AGA TAC CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

             850          860          870          880
TTT AAT CCC ATG GAA TCA ATC AAA TTA AAT CAT CAT GAC TAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

```
          890                900                910                920
ATT GAT TCC CCA TCG CTG AAG GAC AGA ATT CAT TGT GTG GCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

     930                940                950                960
TTT GTA TTT GAT GCC AGC TCT ATT GAA TAC TTC TCC TCT CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

   970                980                990               1000
ATG ATA GTA AAG ATC AAA AGA ATT CGA AGG GAG TTG GTA AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

1010               1020               1030               1040               1050
GCT GGT GTG GTA CAT GTG GCT TTG CTC ACT CAT GTG GAT AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

          1060               1070               1080               1090
ATG GAT CTG ATT ACA AAA GGT GAC CTT ATA GAA ATA GAG AGA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

     1100               1110               1120               1130
TGT GTG CCT GTG AGG TCC AAG CTA GAG GAA GTC CAA AGA AAA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

   1140               1150               1160               1170
CTT GGA TTT GCT CTT TCT GAC ATC TCG GTG GTT AGC AAT TAT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

  1180               1190               1200               1210
TCC TCT GAG TGG GAG CTG GAC CCT GTA AAG GAT GTT CTA ATT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

1220               1230               1240               1250               1260
CTT TCT GCT CTG AGA CGA ATG CTA TGG GCT GCA GAT GAC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

          1270               1280               1290               1300
TTA GAG GAT TTG CCT TTT GAG CAA ATA GGG AAT CTA AGG GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

     1310               1320               1330
GAA ATT ATC AAC TGT GCA CAA GGA AAA AAA 3'
--- --- --- --- --- --- --- --- --- --- 5'
```

dans laquelle le signe "–" représente une base complémentaire de la base représentée juste au-dessus de chaque signe.

3. Vecteur d'expression dans lequel un fragment d'ADN contenant une séquence de bases telle que définie à la revendication 1 ou 2 et codant pour un antigène rencontré spécifiquement chez un hôte atteint de l'hépatite non-A non-B, est introduit dans un site de clonage présent en aval d'un promoteur dudit vecteur.

4. Vecteur d'expression selon la revendication 3, dans lequel le promoteur peut être contrôlé par un facteur de régulation.

5. Vecteur d'expression selon la revendication 3, dans lequel le promoteur opère dans un microorganisme.

6. Vecteur d'expression selon la revendication 3, dans lequel le promoteur opère dans un eucaryote.

7. Transformant obtenu par transformation d'un hôte par un vecteur d'expression dans lequel un fragment d'ADN contenant une séquence de bases telle que définie à la revendication 1 ou 2 et codant pour un antigène

rencontré spécifiquement chez un hôte atteint de l'hépatite non-A non-B, est introduit dans un site de clonage présent en aval d'un promoteur dudit vecteur.

8. Transformant selon la revendication 7, dans lequel l'hôte est Escherichia coli ou Bacillus subtilis.

9. Procédé de production d'un antigène rencontré spécifiquement chez un hôte atteint de l'hépatite non-A non-B, comprenant l'introduction d'un fragment d'ADN contenant une séquence de bases telle que définie à la revendication 1 ou 2 et codant pour ledit antigène rencontré spécifiquement dans un site de clonage présent en aval d'un promoteur d'un vecteur d'expression, l'introduction du vecteur d'expression contenant ledit fragment d'ADN dans un hôte, la culture dudit transformant, et la récupération de l'antigène produit et accumulé.

10. Procédé pour le diagnostic in vitro de l'hépatite non-A non-B, qui comprend la mise en contact d'un échantillon de foie et/ou d'un échantillon de sérum prélevé chez un patient soupçonné être infecté par une hépatite non-A non-B, avec la protéine dont la séquence apparaît à la revendication 1, ou une partie de celle-ci, pendant un laps de temps suffisant et dans des conditions suffisantes pour permettre la production d'un complexe entre ladite protéine ou une partie de celle-ci, avec les anticorps contenus dans l'échantillon du patient, et la détection de la présence du complexe immunologique, en particulier, lorsque le patient souffre d'une hépatite non-A non-B.

Patentansprüche

1. DNA-Fragment, das eine für ein antigenes Protein, das spezifisch in einem mit non-A non-B Hepatitis befallenen Wirt vorkommt, kodierende Basensequenz enthält, wobei das Protein die ganze oder einen Teil der durch die Formel dargestellten Aminosäuresequenz enthält :

```
                                                                  10
Met Ala Val Thr Thr Arg Leu Thr Trp Leu His Glu Lys Ile Leu

                  20                                                  30
Gln Asn His Phe Gly Gly Lys Arg Leu Ser Leu Leu Tyr Lys Gly

                                                                  40
Ser Val His Gly Phe His Asn Gly Val Leu Leu Asp Arg Cys Cys

                  50                                                  60
Asn Gln Gly Pro Thr Leu Thr Val Ile Tyr Ser Glu Asp His Ile

                                                                  70
Ile Gly Ala Tyr Ala Glu Glu Gly Tyr Gln Glu Arg Lys Tyr Ala

                  80                                                  90
Ser Ile Ile Leu Phe Ala Leu Gln Glu Thr Lys Ile Ser Glu Trp

                                                                 100
Lys Leu Gly Leu Tyr Thr Pro Glu Thr Leu Phe Cys Cys Asp Val

                 110                                                 120
Ala Lys Tyr Asn Ser Pro Thr Asn Phe Gln Ile Asp Gly Arg Asn

                                                                 130
Arg Lys Val Ile Met Asp Leu Lys Thr Met Glu Asn Leu Gly Leu

                 140                                                 150
Ala Gln Asn Cys Thr Ile Ser Ile Gln Asp Tyr Glu Val Phe Arg

                                                                 160
Cys Glu Asp Ser Leu Asp Glu Arg Lys Ile Lys Gly Val Ile Glu

                 170                                                 180
Leu Arg Lys Ser Leu Leu Ser Ala Leu Arg Thr Tyr Glu Pro Tyr

                                                                 190
Gly Ser Leu Val Gln Gln Ile Arg Ile Leu Leu Leu Gly Pro Ile
```

```
                                200                                 210
(Gly Ala Gly Lys Ser Ser Phe Phe Asn Ser Val Arg Ser Val Phe

                                                    220
(Gln Gly His Val Thr His Gln Ala Leu Val Gly Thr Asn Thr Thr

                                230                                 240
 Gly Ile Ser Glu Lys Tyr Arg Thr Tyr Ser Ile Arg Asp Gly Lys

                                                    250
 Asp Gly Lys Tyr Leu Pro Phe Ile Leu Cys Asp Ser Leu Gly Leu

                                260                                 270
 Ser Glu Lys Glu Gly Gly Leu Cys Met Asp Asp Ile Ser Tyr Ile

                                                    280
 Leu Asn Gly Asn Ile Arg Asp Arg Tyr Gln Phe Asn Pro Met Glu

                                290                                 300
 Ser Ile Lys Leu Asn His His Asp Tyr Ile Asp Ser Pro Ser Leu

                                                    310
 Lys Asp Arg Ile His Cys Val Ala Phe Val Phe Asp Ala Ser Ser

                                320                                 330
 Ile Glu Tyr Phe Ser Ser Gln Met Ile Val Lys Ile Lys Arg Ile

                                                    340
 Arg Arg Glu Leu Val Asn Ala Gly Val Val His Val Ala Leu Leu

                                350                                 360
 Thr His Val Asp Ser Met Asp Leu Ile Thr Lys Gly Asp Leu Ile

                                                    370
 Glu Ile Glu Arg Cys Val Pro Val Arg Ser Lys Leu Glu Glu Val

                                380                                 390
 Gln Arg Lys Leu Gly Phe Ala Leu Ser Asp Ile Ser Val Val Ser


                                                    400
 Asn Tyr Ser Ser Glu Trp Glu Leu Asp Pro Val Lys Asp Val Leu

                                410                                 420
 Ile Leu Ser Ala Leu Arg Arg Met Leu Trp Ala Ala Asp Asp Phe

                                                    430
 Leu Glu Asp Leu Pro Phe Glu Gln Ile Gly Asn Leu Arg Glu Glu

                                440
 Ile Ile Asn Cys Ala Gln Gly Lys Lys ***.
```

2. DNA-Fragment nach Anspruch 1, bei dem die Basensequenz die ganze oder einen Teil der durch die Formel dargestellten Phasensequenz enthält :

```
                  10                  20                  30                  40
5'  ATG GCA GTG ACA ACT CGT TGG ACA TGG TTG CAT GAA AAG ATC
3'  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                  50                  60                  70                  80
    CTG CAA AAT CAT TTT GGA GGG AAG CGG CTT AGC CTT CTC TAT
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                  90                 100                 110                 120
    AAG GGT AGT GTC CAT GGA TTC CAT AAT GGA GTT TTG CTT GAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                 130                 140                 150                 160
    AGA TGT TGT AAT CAA GGG CCT ACT CTA ACA GTG ATT TAT AGT
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                 170                 180                 190                 200                 210
    GAA GAT CAT ATT ATT GGA GCA TAT GCA GAA GAG GGT TAC CAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                 220                 230                 240                 250
    GAA AGA AAG TAT GCT TCC ATC ATC CTT TTT GCA CTT CAA GAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                 260                 270                 280                 290
    ACT AAA ATT TCA GAA TGG AAA CTA GGA CTA TAT ACA CCA GAA
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                 300                 310                 320                 330
    ACA CTG TTT TGT TGT GAC GTT GCA AAA TAT AAC TCC CCA ACT
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

```
      340                 350                 360                 370
AAT TTC CAG ATA GAT GGA AGA AAT AGA AAA GTG ATT ATG GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

380                 390                 400                 410                 420
TTA AAG ACA ATG GAA AAT CTT GGA CTT GCT CAA AAT TGT ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

            430                 440                 450                 460
ATC TCT ATT CAG GAT TAT GAA GTT TTT CGA TGC GAA GAT TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

         470                 480                 490                 500
CTG GAC GAA AGA AAG ATA AAA GGG GTC ATT GAG CTC AGG AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

      510                 520                 530                 540
AGC TTA CTG TCT GCC TTG AGA ACT TAT GAA CCA TAT GGA TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

   550                 560                 570                 580
CTG GTT CAA CAA ATA CGA ATT CTG CTG CTG GGT CCA ATT GGA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

590                 600                 610                 620                 630
GCT GGG AAG TCT AGC TTT TTC AAC TCA GTG AGG TCT GTT TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

            640                 650                 660                 670
CAA GGG CAT GTA ACG CAT CAG GCT TTG GTG GGC ACT AAT ACA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

         680                 690                 700                 710
ACT GGG ATA TCT GAG AAG TAT AGG ACA TAC TCT ATT AGA GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

      720                 730                 740                 750
GGG AAA GAT GGC AAA TAC CTG CCA TTT ATT CTG TGT GAC TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

   760                 770                 780                 790
CTG GGG CTG AGT GAG AAA GAA GGC GGC CTG TGC ATG GAT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

800                 810                 820                 830                 840
ATA TCC TAC ATC TTG AAC GGT AAC ATT CGT GAT AGA TAC CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

            850                 860                 870                 880
TTT AAT CCC ATG GAA TCA ATC AAA TTA AAT CAT CAT GAC TAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

```
          890                900                910                920
ATT GAT TCC CCA TCG CTG AAG GAC AGA ATT CAT TGT GTG GCA
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

     930                940                950                960
TTT GTA TTT GAT GCC AGC TCT ATT GAA TAC TTC TCC TCT CAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

  970                980                990               1000
ATG ATA GTA AAG ATC AAA AGA ATT CGA AGG GAG TTG GTA AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

1010          1020               1030          1040          1050
  GCT GGT GTG GTA CAT GTG GCT TTG CTC ACT CAT GTG GAT AGC
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

            1060          1070          1080          1090
  ATG GAT CTG ATT ACA AAA GGT GAC CTT ATA GAA ATA GAG AGA
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

        1100          1110          1120          1130
  TGT GTG CCT GTG AGG TCC AAG CTA GAG GAA GTC CAA AGA AAA
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

     1140          1150          1160          1170
  CTT GGA TTT GCT CTT TCT GAC ATC TCG GTG GTT AGC AAT TAT
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

   1180          1190          1200          1210
  TCC TCT GAG TGG GAG CTG GAC CCT GTA AAG GAT GTT CTA ATT
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

1220          1230               1240          1250          1260
  CTT TCT GCT CTG AGA CGA ATG CTA TGG GCT GCA GAT GAC TTC
  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

          1270          1280          1290          1300
TTA GAG GAT TTG CCT TTT GAG CAA ATA GGG AAT CTA AGG GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- ---

        1310          1320          1330
  GAA ATT ATC AAC TGT GCA CAA GGA AAA AAA 3'
  --- --- --- --- --- --- --- --- --- --- 5'
```

wobei "–" jeweils die zur direkt darüberstehenden Base komplementäre Base darstellt.

3. Expressionsvektor, bei dem ein DNA-Fragment, das eine Basensequenz nach Anspruch 1 oder 2 enthält und für ein Antigen kodiert, das spezifisch in einem mit non-A non-B Hepatitis befallenen Wirt auftritt, in eine stromabwärts vom Promotor des Vektors vorhandene Klonierungsstelle eingeführt wird.

4. Expressionsvektor nach Anspruch 3, bei dem der Promotor durch einen Regulationsfaktor kontrollierbar ist.

5. Expressionsvektor nach Anspruch 3, bei dem der Promotor in einem Mikroorganismus wirksam ist.

6. Expressionsvektor nach Anspruch 3, bei dem der Promotor in einem Eukaryonten wirksam ist.

7. Transformante, erhalten durch Transformieren eines Wirtes mit einem Expressionsvektor, bei dem ein DNA-Fragment, das eine Basensequenz nach Anspruch 1 oder 2 enthält, die für ein Antigen kodiert, das spe-

zifisch in einem mit non-A non-B Hepatitis befallenen Wirt auftritt, in eine stromabwärts vom Promotor des Vektors vorhandene Klonierungsstelle eingeführt wird.

8. Transformante nach Anspruch 7, bei der der Wirt Escherichia coli oder Bacillus subtilis ist.

9. Verfahren zur Herstellung eines Antigens, das spezifisch in einem mit non-A non-B Hepatitis befallenen Wirt vorkommt, umfassend die Schritte : Einführen eines DNA-Fragments, das eine Basensequenz nach Anspruch 1 oder 2 enthält, die für das spezifisch vorkommende Antigen kodiert, in eine stromabwärts vom Promotor eines Expressionsvektors vorhandene Klonierungsstelle, Einführen des das DNA-Fragment enthaltenden Expressionsvektors in einen Wirt, Kultivieren der Transformanten und Gewinnung des produzierten und akkumulierten Antigens.

10. Verfahren zur in vitro Diagnose von non-A non-B Hepatitis, umfassend die Schritte : in Kontakt bringen einer Leberprobe und/oder einer Serumprobe, die von einem Patienten mit Verdacht auf eine non-A non-B Hepatitisinfektion entnommen wurde, mit dem Protein mit einer Sequenz wie in Anspruch 1, oder einem Teil davon, für eine bestimmte Zeit und unter Bedingungen, die ausreichend sind für die Komplexbildung zwischen dem Protein, oder einem Teil davon, mit dem in der Patientenprobe enthaltenen Antikörpern, und Nachweis der Anwesenheit des immunologischen Komplexes, insbesondere wenn der Patient an non-A non-B Hepatitis leidet.

Fig. 1a

```
                    10                  20                  30
5' ATG GCA GTG ACA ACT CGT TTG ACA TGG TTG
3'

                    40                  50                  60
CAT GAA AAG ATC CTG CAA AAT CAT TTT GGA

                70                  80                  90
GGG AAG CGG CTT AGC CTT CTC TAT AAG GGT

               100                 110                 120
AGT GTC CAT GGA TTC CAT AAT GGA GTT TTG

               130                 140                 150
CTT GAC AGA TGT TGT AAT CAA GGG CCT ACT

               160                 170                 180
CTA ACA GTG ATT TAT AGT GAA GAT CAT ATT

               190                 200                 210
ATT GGA GCA TAT GCA GAA GAG GGT TAC CAG

               220                 230                 240
GAA AGA AAG TAT GCT TCC ATC ATC CTT TTT

               250                 260                 270
GCA CTT CAA GAG ACT AAA ATT TCA GAA TGG

               280                 290                 300
AAA CTA GGA CTA TAT ACA CCA GAA ACA CTG
```

Fig. 1b

```
                310                 320                 330
TTT TGT TGT GAC GTT GCA AAA TAT AAC TCC

                340                 350                 360
CCA ACT AAT TTC CAG ATA GAT GGA AGA AAT

                370                 380                 390
AGA AAA GTG ATT ATG GAC TTA AAG ACA ATG

                400                 410                 420
GAA AAT CTT GGA CTT GCT CAA AAT TGT ACT

                430                 440                 450
ATC TCT ATT CAG GAT TAT GAA GTT TTT CGA

                460                 470                 480
TGC GAA GAT TCA CTG GAC GAA AGA AAG ATA

                490                 500                 510
AAA GGG GTC ATT GAG CTC AGG AAG AGC TTA

                520                 530                 540
CTG TCT GCC TTG AGA ACT TAT GAA CCA TAT

                550                 560                 570
GGA TCC CTG GTT CAA CAA ATA CGA ATT CTG

                580                 590                 600
CTG CTG GGT CCA ATT GGA GCT GGG AAG TCT
```

Fig. 1c

610 620 630
AGC TTT TTC AAC TCA GTG AGG TCT GTT TTC

640 650 660
CAA GGG CAT GTA ACG CAT CAG GCT TTG GTG

670 680 690
GGC ACT AAT ACA ACT GGG ATA TCT GAG AAG

700 710 720
TAT AGG ACA TAC TCT ATT AGA GAC GGG AAA

730 740 750
GAT GGC AAA TAC CTG CCA TTT ATT CTG TGT

760 770 780
GAC TCA CTG GGG CTG AGT GAG AAA GAA GGC

790 800 810
GGC CTG TGC ATG GAT GAC ATA TCC TAC ATC

820 830 840
TTG AAC GGT AAC ATT CGT GAT AGA TAC CAG

850 860 870
TTT AAT CCC ATG GAA TCA ATC AAA TTA AAT

880 890 900
CAT CAT GAC TAC ATT GAT TCC CCA TCG CTG

Fig. 1d

910 920 930
AAG GAC AGA ATT CAT TGT GTG GCA TTT GTA

940 950 960
TTT GAT GCC AGC TCT ATT GAA TAC TTC TCC

970 980 990
TCT CAG ATG ATA GTA AAG ATC AAA AGA ATT

1000 1010 1020
CGA AGG GAG TTG GTA AAC GCT GGT GTG GTA

1030 1040 1050
CAT GTG GCT TTG CTC ACT CAT GTG GAT AGC

1060 1070 1080
ATG GAT CTG ATT ACA AAA GGT GAC CTT ATA

1090 1100 1110
GAA ATA GAG AGA TGT GTG CCT GTG AGG TCC

1120 1130 1140
AAG CTA GAG GAA GTC CAA AGA AAA CTT GGA

1150 1160 1170
TTT GCT CTT TCT GAC ATC TCG GTG GTT AGC

1180 1190 1200
AAT TAT TCC TCT GAG TGG GAG CTG GAC CCT

Fig. 1e

```
          1210          1220          1230
GTA AAG GAT GTT CTA ATT CTT TCT GCT CTG

          1240          1250          1260
AGA CGA ATG CTA TGG GCT GCA GAT GAC TTC

          1270          1280          1290
TTA GAG GAT TTG CCT TTT GAG CAA ATA GGG

          1300          1310          1320
AAT CTA AGG GAG GAA ATT ATC AAC TGT GCA

          1330
CAA GGA AAA AAA 3'
                5'
```

Fig. 2

AAAAATTTATTTGCTTTCAGGAAAATTTTTCTGT
TTTTTAAATAAACGAAAGTCCTTTTAAAAAGACA

ATAATGTGTGGAATTGTGAGCGGATAACAATTTC
TATTACACACCTTAACACTCGCCTATTGTTAAAG

Fig. 3a

```
          250         260         270         280         290         300
CTA GGA CTA TAT ACA CCA GAA ACA CTG TTT TGT TGT GAC GTT GCA AAA TAT AAC TCC CCA
Leu Gly Leu Tyr Thr Pro Glu Thr Leu Phe Cys Cys Asp Val Ala Lys Tyr Asn Ser Pro

          310         320         330         340         350         360
ACT AAT TTC CAG ATA GAT GGA AGA AAT AGA AAA GTG ATT ATG GAC TTA AAG ACA ATG GAA
Thr Asn Phe Gln Ile Asp Gly Arg Asn Arg Lys Val Ile Met Asp Leu Lys Thr Met Glu

          370         380         390         400         410         420
AAT CTT GGA CTT GCT CAA AAT TGT ACT ATC TCT ATT CAG GAT TAT GAA GTT TTT CGA TGC
Asn Leu Gly Leu Ala Gln Asn Cys Thr Ile Ser Ile Gln Asp Tyr Glu Val Phe Arg Cys

          430         440         450         460         470         480
GAA GAT TCA CTG GAC GAA AGA AAG ATA AAA GGG GTC ATT GAG CTC AGG AAG AGC TTA CTG
Glu Asp Ser Leu Asp Glu Arg Lys Ile Lys Gly Val Ile Glu Leu Arg Lys Ser Leu Leu

          490         500         510         520         530         540
TCT GCC TTG AGA ACT TAT GAA CCA TAT GGA TCC CTG GTT CAA CAA ATA CGA ATT CTG CTG
Ser Ala Leu Arg Thr Tyr Glu Pro Tyr Gly Ser Leu Val Gln Gln Ile Arg Ile Leu Leu

          550         560         570         580         590         600
CTG GGT CCA ATT GGA GCT GGG AAG TCT AGC TTT TTC AAC TCA GTG AGG TCT GTT TTC CAA
Leu Gly Pro Ile Gly Ala Gly Lys Ser Ser Phe Phe Asn Ser Val Arg Ser Val Phe Gln
```

Fig. 3b

```
          610         620         630         640         650         660
GGG CAT GTA ACG CAT CAG GCT TTG GTG GGC ACT AAT ACA ACT GGG ATA TCT GAG AAG TAT
Gly His Val Thr His Gln Ala Leu Val Gly Thr Asn Thr Thr Gly Ile Ser Glu Lys Tyr

          670         680         690         700         710         720
AGG ACA TAC TCT ATT AGA GAC GGG AAA GAT GGC AAA TAC CTG CCA TTT ATT CTG TGT GAC
Arg Thr Tyr Ser Ile Arg Asp Gly Lys Asp Gly Lys Tyr Leu Pro Phe Ile Leu Cys Asp

          730         740         750         760         770         780
TCA CTG GGG CTG AGT GAG AAA GAA GGC GGC CTG TGC ATG GAT GAC ATA TCC TAC ATC TTG
Ser Leu Gly Leu Ser Glu Lys Glu Gly Gly Leu Cys Met Asp Asp Ile Ser Tyr Ile Leu

          790         800         810         820         830         840
AAC GGT AAC ATT CGT GAT AGA TAC CAG TTT AAT CCC ATG GAA TCA ATC AAA TTA AAT CAT
Asn Gly Asn Ile Arg Asp Arg Tyr Gln Phe Asn Pro Met Glu Ser Ile Lys Leu Asn His

          850         860         870         880         890         900
CAT GAC TAC ATT GAT TCC CCA TCG CTG AAG GAC AGA ATT CAT TGT GTG GCA TTT GTA TTT
His Asp Tyr Ile Asp Ser Pro Ser Leu Lys Asp Arg Ile His Cys Val Ala Phe Val Phe

          910         920         930         940         950         960
GAT GCC AGC TCT ATT GAA TAC TTC TCC TCT CAG ATG ATA GTA AAG ATC AAA AGA ATT CGA
Asp Ala Ser Ser Ile Glu Tyr Phe Ser Ser Gln Met Ile Val Lys Ile Lys Arg Ile Arg

          970         980         990         1000        1010        1020
AGG GAG TTG GTA AAC GCT GGT GTG GTA CAT GTG GCT TTG CTC ACT CAT GTG GAT AGC ATG
Arg Glu Leu Val Asn Ala Gly Val Val His Val Ala Leu Leu Thr His Val Asp Ser Met
```


EP 0 293 274 B1

Fig. 3c

```
          1030          1040          1050          1060          1070          1080
GAT CTG ATT ACA AAA GGT GAC CTT ATA GAA ATA GAG AGA TGT GTG CCT GTG AGG TCC AAG
Asp Leu Ile Thr Lys Gly Asp Leu Ile Glu Ile Glu Arg Cys Val Pro Val Arg Ser Lys

          1090          1100          1110          1120          1130          1140
CTA GAG GAA GTC CAA AGA AAA CTT GGA TTT GCT CTT TCT GAC ATC TCG GTG GTT AGC AAT
Leu Glu Glu Val Gln Arg Lys Leu Gly Phe Ala Leu Ser Asp Ile Ser Val Val Ser Asn

          1150          1160          1170          1180          1190          1200
TAT TCC TCT GAG TGG GAG CTG GAC CCT GTA AAG GAT GTT CTA ATT CTT TCT GCT CTG AGA
Tyr Ser Ser Glu Trp Glu Leu Asp Pro Val Lys Asp Val Leu Ile Leu Ser Ala Leu Arg

          1210          1220          1230          1240          1250          1260
CGA ATG CTA TGG GCT GCA GAT GAC TTC TTA GAG GAT TTG CCT TTT GAG CAA ATA GGG AAT
Arg Met Leu Trp Ala Ala Asp Asp Phe Leu Glu Asp Leu Pro Phe Glu Gln Ile Gly Asn

          1270          1280          1290          1300
CTA AGG GAG GAA ATT ATC AAC TGT GCA CAA GGA AAA AAA TAG
Leu Arg Glu Glu Ile Ile Asn Cys Ala Gln Gly Lys Lys ***
```


EP 0 293 274 B1

Fig. 4a

```
          10         20         30         40         50         60         70         80
5' GGGGGGCTAC CCTCAGCTCT AGCTCATACT ACAGACAGTA CAACAGATCA AGAAGTATGG CAGTGACAAC TCGTTTGACA
3' CCCCCCGATG GGAGTCGAGA TCGAGTATGA TGTCTGTCAT GTTGTCTAGT TCTTCATACC GTCACTGTTG AGCAAACTGT

          90         100        110        120        130        140        150        160
   TGGTTGCATG AAAAGATCCT GCAAAATCAT TTTGGAGGGA AGCGGCTTAG CCTTCTCTAT AAGGGTAGTG TCCATGGATT
   ACCAACGTAC TTTTCTAGGA CGTTTTAGTA AAACCTCCCT TCGCCGAATC GGAAGAGATA TTCCCATCAC AGGTACCTAA

         170        180        190        200        210        220        230        240
   CCATAATGGA GTTTTGCTTG ACAGATGTTG TAATCAAGGG CCTACTCTAA CAGTGATTTA TAGTGAAGAT CATATTATTG
   GGTATTACCT CAAAACGAAC TGTCTACAAC ATTAGTTCCC GGATGAGATT GTCACTAAAT ATCACTTCTA GTATAATAAC

         250        260        270        280        290        300        310        320
   GAGCATATGC AGAAGAGGGT TACCAGGAAA GAAAGTATGC TTCCATCATC CTTTTTGCAC TTCAAGAGAC TAAAATTTCA
   CTCGTATACG TCTTCTCCCA ATGGTCCTTT CTTTCATACG AAGGTAGTAG GAAAAACGTG AAGTTCTCTG ATTTTAAAGT

         330        340        350        360        370        380        390        400
   GAATGGAAAC TAGGACTATA TACACCAGAA ACACTGTTTT GTTGTGACGT TGCAAAATAT AACTCCCCAA CTAATTTCCA
   CTTACCTTTG ATCCTGATAT ATGTGGTCTT TGTGACAAAA CAACACTGCA ACGTTTTATA TTGAGGGGTT GATTAAAGGT

         410        420        430        440        450        460        470        480
   GATAGATGGA AGAAATAGAA AAGTGATTAT GGACTTAAAG ACAATGGAAA ATCTTGGACT TGCTCAAAAT TGTACTATCT
   CTATCTACCT TCTTTATCTT TTCACTAATA CCTGAATTTC TGTTACCTTT TAGAACCTGA ACGAGTTTTA ACATGATAGA

         490        500        510        520        530        540        550        560
   CTATTCAGGA TTATGAAGTT TTTCGATGCG AAGATTCACT GGACGAAAGA AAGATAAAAG GGGTCATTGA GCTCAGGAAG
   GATAAGTCCT AATACTTCAA AAAGCTACGC TTCTAAGTGA CCTGCTTTCT TTCTATTTTC CCCAGTAACT CGAGTCCTTC
```

Fig. 4b

```
       570        580        590        600        610        620        630        640
AGCTTACTGT CTGCCTTGAG AACTTATGAA CCATATGGAT CCCTGGTTCA ACAAATACGA ATTCTGCTGC TGGGTCCAAT
TCGAATGACA GACGGAACTC TTGAATACTT GGTATACCTA GGGACCAAGT TGTTTATGCT TAAGACGACG ACCCAGGTTA

       650        660        670        680        690        700        710        720
TGGAGCTGGG AAGTCTAGCT TTTTCAACTC AGTGAGGTCT GTTTTCCAAG GGCATGTAAC GCATCAGGCT TTGGTGGGCA
ACCTCGACCC TTCAGATCGA AAAAGTTGAG TCACTCCAGA CAAAAGGTTC CCGTACATTG CGTAGTCCGA AACCACCCGT

       730        740        750        760        770        780        790        800
CTAATACAAC TGGGATATCT GAGAAGTATA GGACATACTC TATTAGAGAC GGGAAAGATG GCAAATACCT GCCATTTATT
GATTATGTTG ACCCTATAGA CTCTTCATAT CCTGTATGAG ATAATCTCTG CCCTTTCTAC CGTTTATGGA CGGTAAATAA

       810        820        830        840        850        860        870        880
CTGTGTGACT CACTGGGGCT GAGTGAGAAA GAAGGCGGCC TGTGCATGGA TGACATATCC TACATCTTGA ACGGTAACAT
GACACACTGA GTGACCCCGA CTCACTCTTT CTTCCGCCGG ACACGTACCT ACTGTATAGG ATGTAGAACT TGCCATTGTA

       890        900        910        920        930        940        950        960
TCGTGATAGA TACCAGTTTA ATCCCATGGA ATCAATCAAA TTAAATCATC ATGACTACAT TGATTCCCCA TCGCTGAAGG
AGCACTATCT ATGGTCAAAT TAGGGTACCT TAGTTAGTTT AATTTAGTAG TACTGATGTA ACTAAGGGGT AGCGACTTCC

       970        980        990       1000       1010       1020       1030       1040
ACAGAATTCA TTGTGTGGCA TTTGTATTTG ATGCCAGCTC TATTGAATAC TTCTCCTCTC AGATGATAGT AAAGATCAAA
TGTCTTAAGT AACACACCGT AAACATAAAC TACGGTCGAG ATAACTTATG AAGAGGAGAG TCTACTATCA TTTCTAGTTT

      1050       1060       1070       1080       1090       1100       1110       1120
AGAATTCGAA GGGAGTTGGT AAACGCTGGT GTGGTACATG TGGCTTTGCT CACTCATGTG GATAGCATGG ATCTGATTAC
TCTTAAGCTT CCCTCAACCA TTTGCGACCA CACCATGTAC ACCGAAACGA GTGAGTACAC CTATCGTACC TAGACTAATG
```


EP 0 293 274 B1

Fig. 4c

```
      1130        1140        1150        1160        1170        1180        1190        1200
AAAAGGTGAC CTTATAGAAA TAGAGAGATG TGTGCCTGTG AGGTCCAAGC TAGAGGAAGT CCAAAGAAAA CTTGGATTTG
TTTTCCACTG GAATATCTTT ATCTCTCTAC ACACGGACAC TCCAGGTTCG ATCTCCTTCA GGTTTCTTTT GAACCTAAAC

      1210        1220        1230        1240        1250        1260        1270        1280
CTCTTTCTGA CATCTCGGTG GTTAGCAATT ATTCCTCTGA GTGGGAGCTG GACCCTGTAA AGGATGTTCT AATTCTTTCT
GAGAAAGACT GTAGAGCCAC CAATCGTTAA TAAGGAGACT CACCCTCGAC CTGGGACATT TCCTACAAGA TTAAGAAAGA

      1290        1300        1310        1320        1330        1340        1350        1360
GCTCTGAGAC GAATGCTATG GGCTGCAGAT GACTTCTTAG AGGATTTGCC TTTTGAGCAA ATAGGGAATC TAAGGGAGGA
CGAGACTCTG CTTACGATAC CCGACGTCTA CTGAAGAATC TCCTAAACGG AAAACTCGTT TATCCCTTAG ATTCCCTCCT

      1370        1380        1390        1400        1410        1420        1430        1440
AATTATCAAC TGTGCACAAG GAAAAAAATA GATATGTGAA AGGTTCACGT AAATTTCCTC ACATCACAGA AGATTAAAAT
TTAATAGTTG ACACGTGTTC CTTTTTTTAT CTATACACTT TCCAAGTGCA TTTAAAGGAG TGTAGTGTCT TCTAATTTTA

      1450        1460        1470        1480        1490        1500        1510        1520
TCAGAAAGGA GAAAACACAG ACCAAAGAGA AGTAACTAAG ACCAAAGGGA TGTGTTTTAT TAATGTCTAG GATGAAGAAA
AGTCTTTCCT CTTTTGTGTC TGGTTTCTCT TCATTGATTC TGGTTTCCCT ACACAAAATA ATTACAGATC CTACTTCTTT

      1530        1540        1550        1560        1570        1580        1590        1600
TGCATAGAAC ATTGTAGTAC TTGTAAATAA CTAGAAATAA CATGATTTAG TCATAATTGT GAAAAATAAT AATAATTTTT
ACGTATCTTG TAACATCATG AACATTTATT GATCTTTATT GTACTAAATC AGTATTAACA CTTTTTATTA TTATTAAAAA

      1610        1620        1630        1640        1650        1660
CTTGGATTTA TGTTCTGTAT CTGTGAAAAA ATAAATTTCT TATAAAAAAA AAAAAAAAAA AAAAAAA 3'
GAACCTAAAT ACAAGACATA GACACTTTTT TATTTAAAGA ATATTTTTTT TTTTTTTTTT TTTTTTT 5'
```

Fig. 5

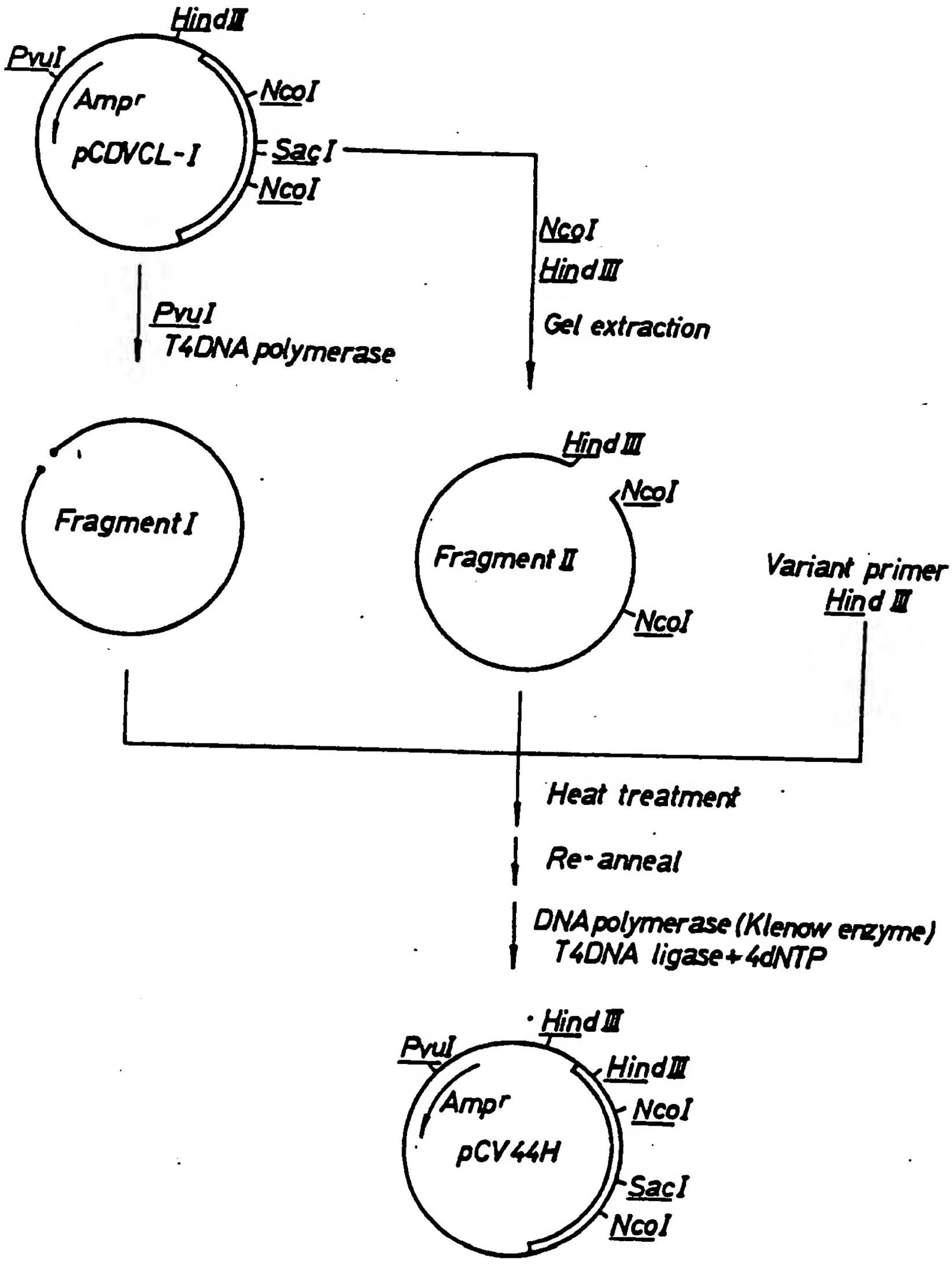

HindIII
PvuI
Ampr
pCDVCL-I
NcoI
SacI
NcoI
NcoI
HindIII
Gel extraction
PvuI
T4DNA polymerase
Fragment I
HindIII
NcoI
Fragment II
NcoI
Variant primer
HindIII
Heat treatment
Re-anneal
DNA polymerase (Klenow enzyme)
T4DNA ligase+4dNTP
HindIII
PvuI
HindIII
Ampr
NcoI
pCV44H
SacI
NcoI

Fig.6

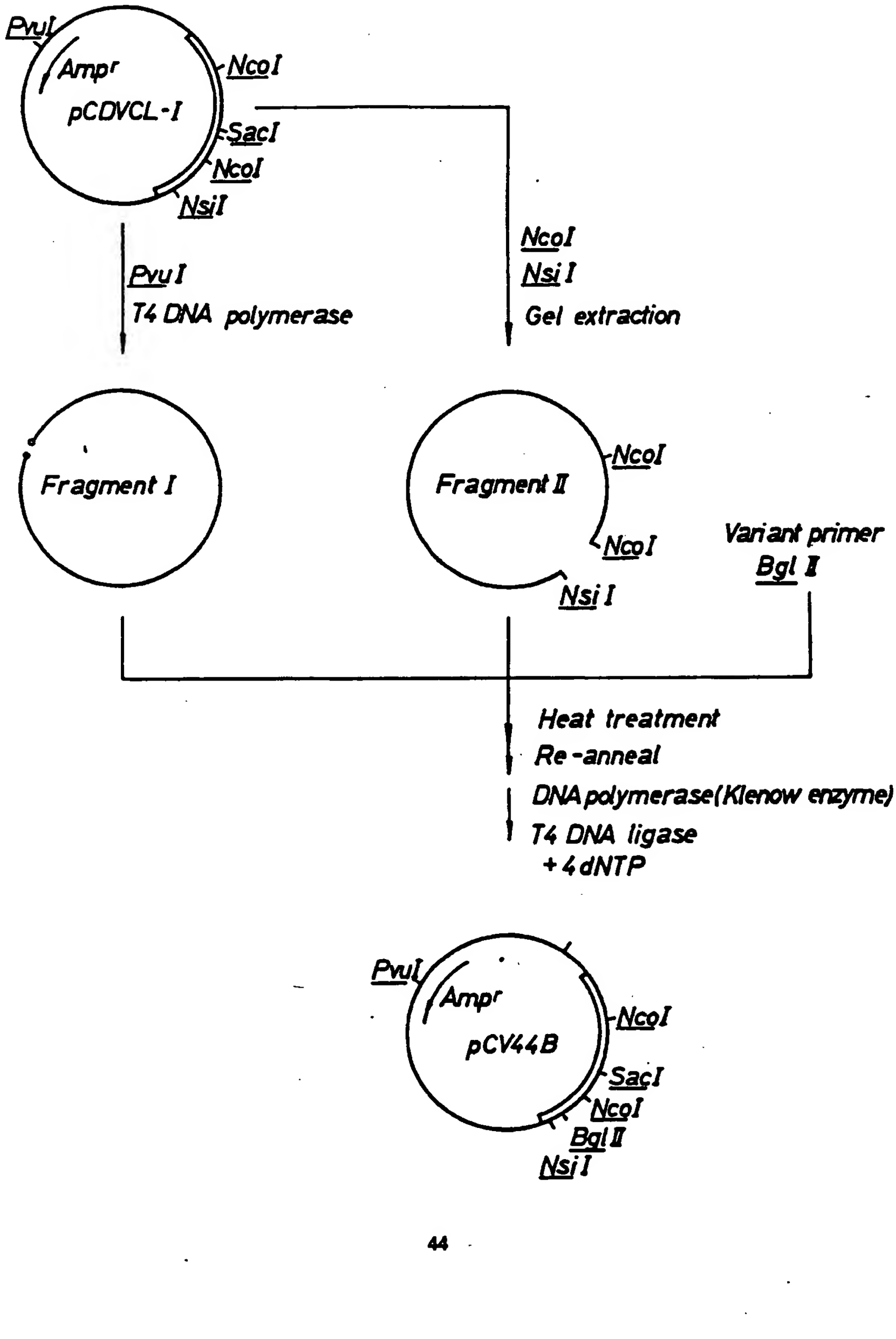
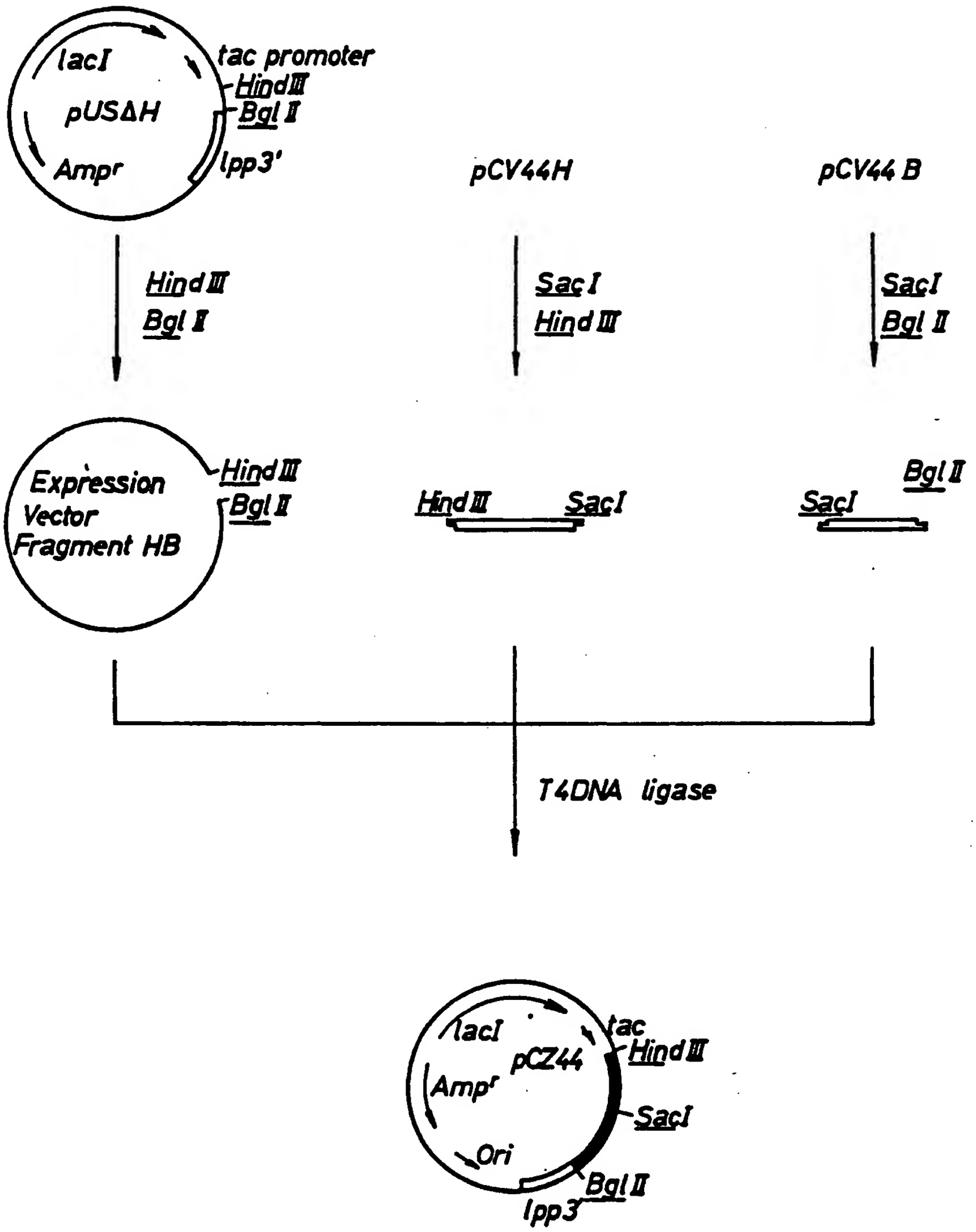

PvuI
Ampr
pCDVCL-I
NcoI
SacI
NcoI
NsiI
PvuI
T4 DNA polymerase
NcoI
NsiI
Gel extraction
Fragment I
Fragment II
NcoI
NcoI
NsiI
Variant primer
BglII
Heat treatment
Re-anneal
DNA polymerase(Klenow enzyme)
T4 DNA ligase
+4dNTP
PvuI
Ampr
pCV44B
NcoI
SacI
NcoI
BglII
NsiI

Fig.7


lacI
tac promoter
HindIII
pUSΔH
BglII
Ampr
lpp3'
pCV44H
pCV44 B
HindIII
BglII
SacI
HindIII
SacI
SacI
BglII
Expression
Vector
Fragment HB
HindIII
BglII
HindIII
SacI
SacI
BglII
T4DNA ligase
lacI
tac
HindIII
pCZ44
Ampr
SacI
Ori
BglII
lpp3